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<b>(54) Title:</b> TRANSFORMING GROWTH FACTOR ALPHA HII  <b>(57) Abstract</b>  The present invention discloses transforming growth factor alpha HII polypeptides and polynucleotides encoding such polypeptides. Also provided is a procedure for producing such polypeptides by recombinant techniques and therapeutic uses of the polypeptides which include stimulating wound healing, treating neurological disorders, treating ocular disorders, treating kidney and liver disorders and stimulating embryogenesis and angiogenesis. Also disclosed are antagonists against such polypeptide and their use as a therapeutic to treat neoplasia. Also disclosed are diagnostic assays for detecting altered levels of the polypeptide of the present invention and mutations in the nucleic acid sequences which encode the polypeptides of the present invention.		

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## TRANSFORMING GROWTH FACTOR ALPHA HII

This invention relates to newly identified polynucleotides, polypeptides encoded by such polynucleotides, the use of such polynucleotides and polypeptides, as well as the production of such polynucleotides and polypeptides. The polypeptide of the present invention has been putatively identified as a human transforming growth factor alpha homolog. More particularly, the polypeptide of the present invention has been putatively identified as transforming growth factor alpha HII, sometimes hereafter referred to as "TGF $\alpha$ -HII". The invention also relates to inhibiting the action of such polypeptides.

Cellular growth and differentiation appear to be initiated, promoted, maintained and regulated by a multiplicity of stimulatory, inhibitory and synergistic factors and hormones. The alteration and/or breakdown of the cellular homeostasis mechanism seems to be a fundamental cause of growth related diseases, including neoplasia. Growth modular factors are implicated in a wide variety of pathological and physiological processes including signal transduction, cell communication, growth and development, embryogenesis, immune response, hematopoiesis cell survival and differentiation, inflammation, tissue repair and remodeling, atherosclerosis and cancer. Epidermal growth

factor (EGF), transforming growth factor alpha (TGF $\alpha$ ), betacellulin, amphiregulin, and vaccinia growth factor among other factors are growth and differentiation modulatory proteins produced by a variety of cell types either under normal physiological conditions or in response to exogenous stimuli and are members of the EGF family.

These peptide growth factors influence wound cells through autocrine and paracrine mechanisms. They also play important roles in normal wound healing in tissues such as skin, cornea and gastrointestinal tract and all share substantial amino acid sequence homology including the conserved placement of three intra-chain disulfide bonds. In addition, all the factors of this family bind to a 170,000 molecular weight transmembrane glycoprotein receptor and activate the tyrosine kinase activity in the receptor's cytoplasmic domain (Buhrow, S.A. et al., J.Bio.Chem., 258:7824-7826 (1983)).

The receptors are expressed by many types of cells including skin keratinocytes, fibroblasts, vascular endothelial cells, and epithelial cells of the GI tract. These peptide growth factors are synthesized by several cells involved in wound healing including platelets, keratinocytes, and activated macrophages. These growth factors have also been implicated in both the stimulation of growth and differentiation of certain cells, for example, neoplasia, and the inhibition of other types of cells.

Betacellulin is a 32-kilodalton glycoprotein that appears to be processed from a larger transmembrane precursor by proteolytic cleavage. The carboxyl-terminal domain of betacellulin has 50% sequence similarity with that of rat transforming growth factor  $\alpha$ . Betacellulin is a potent mitogen for retinal pigment epithelial cells and vascular smooth muscle cells.

Amphiregulin is a bifunctional cell growth regulatory factor which exhibits potent inhibitory activity on DNA

synthesis in neoplastic cells, yet promotes the growth of certain normal cells. A wide variety of uses for amphiregulin have been assigned including the treatment of wounds and cancers. For example, amphiregulin has potent anti-proliferative effects in vitro on several human cancer cell lines of epithelial origin. Amphiregulin also induces the proliferation of human foreskin fibroblasts as shown in United States Patent Application No. 5,115,096.

TGF $\alpha$  has pleiotropic biological effects. The production of certain members of TGF $\alpha$  is synthesized by a number of oncogenically transformed fibroblasts (Ciardiello et al., J.Cell.Biochem., 42:45-57 (1990)), as well as by a variety of tumors, including renal, breast and squamous carcinomas, melanomas and glioblastomas (Derynck, R. et al., Cancer Res., 47:707-712 (1987)). There is direct evidence that TGF $\alpha$  expression can be a contributing factor in the conversion of a normal cell to its tumorigenic counterpart by analyzing transgenic mice in which tumor cells express high levels of TGF $\alpha$ . TGF $\alpha$  transgenic animals display a variety of neoplastic lesions, depending on the strain of mouse and the choice of promotor regulating TGF $\alpha$  expression (Sandgren, et al., Cell, 61:1121-1135 (1990)).

TGF $\alpha$  also plays a role in normal embryonic development and adult physiology (Derynck, R. Adv.Cancer Res., 58:27-5 (1992)). TGF $\alpha$  has been expressed in many tissues including skin, brain, gastrointestinal mucosa and activating macrophages. Accordingly, TGF $\alpha$  is an important factor in controlling growth of epithelial cells and has a role in wound healing. TGF $\alpha$  has also been found to be angiogenic (Schreiber, et al., Science, 232:1250-1253 (1986)).

The polypeptide of the present invention has been putatively identified as transforming growth factor TGF $\alpha$ -HII. This identification has been made as a result of amino acid sequence homology to human TGF $\alpha$ .

In accordance with one aspect of the present invention, there are provided novel mature polypeptides, as well as biologically active and diagnostically or therapeutically useful fragments, analogs and derivatives thereof. The polypeptides of the present invention are of human origin.

In accordance with another aspect of the present invention, there are provided isolated nucleic acid molecules encoding the polypeptides of the present invention, including mRNAs, DNAs, cDNAs, genomic DNAs as well as analogs and biologically active and diagnostically or therapeutically useful fragments thereof.

In accordance with yet a further aspect of the present invention, there are provided processes for producing such polypeptide by recombinant techniques comprising culturing recombinant prokaryotic and/or eukaryotic host cells, containing a nucleic acid sequence encoding a polypeptide of the present invention.

In accordance with yet a further aspect of the present invention, there are provided processes for utilizing such polypeptides, or polynucleotides encoding such polypeptides for therapeutic purposes, for example, to stimulate wound healing to restore normal neurological functioning after trauma or AIDS dementia, to treat ocular disorders, to target certain cells, to treat kidney and liver disorders and to promote hair follicular development, to stimulate angiogenesis for the treatment of burns, ulcers and corneal incisions and to stimulate embryogenesis.

In accordance with yet a further aspect of the present invention, there is also provided nucleic acid probes comprising nucleic acid molecules of sufficient length to specifically hybridize to nucleic acid sequences of the present invention.

In accordance with yet a further aspect of the present invention, there are provided antibodies against such polypeptides.

In accordance with yet a further aspect of the present invention, there are provided agonists to the polypeptide of the present invention.

In accordance with yet another aspect of the present invention, there are provided antagonists to such polypeptides, which may be used to inhibit the action of such polypeptides, for example, in the treatment of corneal inflammation, neoplasia, for example, tumors and cancers and for psoriasis.

In accordance with still another aspect of the present invention, there are provided diagnostic assays for detecting diseases related to overexpression of the polypeptide of the present invention and mutations in the nucleic acid sequences encoding such polypeptide.

In accordance with yet a further aspect of the present invention, there is provided a process for utilizing such polypeptides, or polynucleotides encoding such polypeptides, for in vitro purposes related to scientific research, synthesis of DNA and manufacture of DNA vectors.

These and other aspects of the present invention should be apparent to those skilled in the art from the teachings herein.

The following drawings are illustrative of embodiments of the invention and are not meant to limit the scope of the invention as encompassed by the claims.

Figure 1 depicts the cDNA sequence in corresponding deduced amino acid sequence of TGF $\alpha$ -HII. The standard one letter abbreviations for amino acids are used. The putative signal sequence has been underlined.

Figure 2 is an illustration of comparative amino acid sequence homology between human betacellulin, human TGF $\alpha$  and human TGF $\alpha$ -HII (third row). An \* denotes the conserved EGF motif which is shown to be conserved in the polypeptide of the present invention. Underlining denotes the mature sequence of human TGF $\alpha$ .

In accordance with an aspect of the present invention, there is provided an isolated nucleic acid (polynucleotide) which encodes for the mature polypeptide having the deduced amino acid sequence of Figure 1 (SEQ ID NO:2) or for the mature polypeptide encoded by the cDNA of the clone deposited as ATCC Deposit No. \_\_\_\_\_, on May 15, 1995.

A polynucleotide encoding a polypeptide of the present invention may be obtained from human brain and early stage brain tissue. The polynucleotide of this invention was discovered in a cDNA library derived from two-week old embryo. It is structurally related to the EGF family. It contains an open reading frame encoding a protein of 374 amino acid residues of which approximately the 45 amino acids residues are the putative leader sequence. The protein exhibits the highest degree of homology to human TGF $\alpha$  with 26 % identity and 46 % similarity over a 236 amino acid stretch. TGF $\alpha$ -HII contains all six of the conserved cysteine residues found in all members of EGF family.

The full-length polypeptide of the present invention as set forth in SEQ ID NO:2 has a putative signal sequence which comprises amino acid 1 through amino acid 45 of SEQ ID NO:2 which aids in secretion of the polypeptide from the cell. The polypeptide is further processed wherein amino acid 46 through amino acid 214 of SEQ ID NO:2 are cleaved from the polypeptide since this stretch of amino acids is a putative precursor sequence. Further, amino acid 264 through amino acid 344 represent a putative transmembrane portion which is thought to be necessary to direct the polypeptide to particular target locations for the carrying out of biological functions as hereinafter described. The transmembrane portion may also be cleaved from the polypeptide such that the putative soluble portion of the polypeptide of the present invention comprises amino acid 214 through amino acid 264 of SEQ ID NO:2.



The polynucleotide of the present invention may be in the form of RNA or in the form of DNA, which DNA includes cDNA, genomic DNA, and synthetic DNA. The DNA may be double-stranded or single-stranded, and if single stranded may be the coding strand or non-coding (anti-sense) strand. The coding sequence which encodes the mature polypeptide may be identical to the coding sequence shown in Figure 1 (SEQ ID NO:1) or that of the deposited clone or may be a different coding sequence which coding sequence, as a result of the redundancy or degeneracy of the genetic code, encodes the same mature polypeptide as the DNA of Figure 1 (SEQ ID NO:1) or the deposited cDNA.

The polynucleotide which encodes for the mature polypeptide of Figure 1 (SEQ ID NO:2) or for the mature polypeptide encoded by the deposited cDNA may include, but is not limited to: only the coding sequence for the mature polypeptide; the coding sequence for the mature polypeptide and additional coding sequence such as a leader or secretory sequence or a proprotein sequence; the coding sequence for the mature polypeptide (and optionally additional coding sequence) and non-coding sequence, such as introns or non-coding sequence 5' and/or 3' of the coding sequence for the mature polypeptide.

Thus, the term "polynucleotide encoding a polypeptide" encompasses a polynucleotide which includes only coding sequence for the polypeptide as well as a polynucleotide which includes additional coding and/or non-coding sequence.

The present invention further relates to variants of the hereinabove described polynucleotides which encode for fragments, analogs and derivatives of the polypeptide having the deduced amino acid sequence of Figure 1 (SEQ ID NO:2) or the polypeptide encoded by the cDNA of the deposited clone. The variant of the polynucleotide may be a naturally occurring allelic variant of the polynucleotide or a non-naturally occurring variant of the polynucleotide.

Thus, the present invention includes polynucleotides encoding the same mature polypeptide as shown in Figure 1 (SEQ ID NO:2) or the same mature polypeptide encoded by the cDNA of the deposited clone as well as variants of such polynucleotides which variants encode for a fragment, derivative or analog of the polypeptide of Figure 1 (SEQ ID NO:2) or the polypeptide encoded by the cDNA of the deposited clone. Such nucleotide variants include deletion variants, substitution variants and addition or insertion variants.

As hereinabove indicated, the polynucleotide may have a coding sequence which is a naturally occurring allelic variant of the coding sequence shown in Figure 1 (SEQ ID NO:1) or of the coding sequence of the deposited clone. As known in the art, an allelic variant is an alternate form of a polynucleotide sequence which may have a substitution, deletion or addition of one or more nucleotides, which does not substantially alter the function of the encoded polypeptide.

The present invention also includes polynucleotides, wherein the coding sequence for the mature polypeptide may be fused in the same reading frame to a polynucleotide sequence which aids in expression and secretion of a polypeptide from a host cell, for example, a leader sequence which functions as a secretory sequence for controlling transport of a polypeptide from the cell. The polypeptide having a leader sequence is a preprotein and may have the leader sequence cleaved by the host cell to form the mature form of the polypeptide. The polynucleotides may also encode for a proprotein which is the mature protein plus additional 5' amino acid residues. A mature protein having a prosequence is a proprotein and is an inactive form of the protein. Once the prosequence is cleaved an active mature protein remains.

Thus, for example, the polynucleotide of the present invention may encode for a mature protein, or for a protein

having a prosequence or for a protein having both a prosequence and a presequence (leader sequence).

The polynucleotides of the present invention may also have the coding sequence fused in frame to a marker sequence which allows for purification of the polypeptide of the present invention. The marker sequence may be a hexahistidine tag supplied by a pQE-9 vector to provide for purification of the mature polypeptide fused to the marker in the case of a bacterial host, or, for example, the marker sequence may be a hemagglutinin (HA) tag when a mammalian host, e.g. COS-7 cells, is used. The HA tag corresponds to an epitope derived from the influenza hemagglutinin protein (Wilson, I., et al., Cell, 37:767 (1984)).

The term "gene" means the segment of DNA involved in producing a polypeptide chain; it includes regions preceding and following the coding region (leader and trailer) as well as intervening sequences (introns) between individual coding segments (exons).

Fragments of the full length TGF $\alpha$ -HII gene may be used as a hybridization probe for a cDNA library to isolate the full length gene and to isolate other genes which have a high sequence similarity to the gene or similar biological activity. Probes of this type preferably have at least 30 bases and may contain, for example, 50 or more bases. The probe may also be used to identify a cDNA clone corresponding to a full length transcript and a genomic clone or clones that contain the complete TGF $\alpha$ -HII gene including regulatory and promotor regions, exons, and introns. An example of a screen comprises isolating the coding region of the gene by using the known DNA sequence to synthesize an oligonucleotide probe. Labeled oligonucleotides having a sequence complementary to that of the gene of the present invention are used to screen a library of human cDNA, genomic DNA or mRNA to determine which members of the library the probe hybridizes to.

The present invention further relates to polynucleotides which hybridize to the hereinabove-described sequences if there is at least 70%, preferably at least 90%, and more preferably at least 95% identity between the sequences. The present invention particularly relates to polynucleotides which hybridize under stringent conditions to the hereinabove-described polynucleotides. As herein used, the term "stringent conditions" means hybridization will occur only if there is at least 95% and preferably at least 97% identity between the sequences. The polynucleotides which hybridize to the hereinabove described polynucleotides in a preferred embodiment encode polypeptides which either retain substantially the same biological function or activity as the mature polypeptide encoded by the cDNAs of Figure 1 (SEQ ID NO:1) or the deposited cDNA(s).

Alternatively, the polynucleotide may have at least 20 bases, preferably 30 bases, and more preferably at least 50 bases which hybridize to a polynucleotide of the present invention and which has an identity thereto, as hereinabove described, and which may or may not retain activity. For example, such polynucleotides may be employed as probes for the polynucleotide of SEQ ID NO:1, for example, for recovery of the polynucleotide or as a diagnostic probe or as a PCR primer.

Thus, the present invention is directed to polynucleotides having at least a 70% identity, preferably at least 90% and more preferably at least a 95% identity to a polynucleotide which encodes the polypeptide of SEQ ID NO:2 as well as fragments thereof, which fragments have at least 30 bases and preferably at least 50 bases and to polypeptides encoded by such polynucleotides.

The deposit(s) referred to herein will be maintained under the terms of the Budapest Treaty on the International Recognition of the Deposit of Micro-organisms for purposes of Patent Procedure. These deposits are provided merely as

convenience to those of skill in the art and are not an admission that a deposit is required under 35 U.S.C. §112. The sequence of the polynucleotides contained in the deposited materials, as well as the amino acid sequence of the polypeptides encoded thereby, are incorporated herein by reference and are controlling in the event of any conflict with any description of sequences herein. A license may be required to make, use or sell the deposited materials, and no such license is hereby granted.

The present invention further relates to a polypeptide which has the deduced amino acid sequence of Figure 1 (SEQ ID NO:2) or which has the amino acid sequence encoded by the deposited cDNA, as well as fragments, analogs and derivatives of such polypeptide.

The terms "fragment," "derivative" and "analog" when referring to the polypeptide of Figure 1 (SEQ ID NO:2) or that encoded by the deposited cDNA, means a polypeptide which retains essentially the same biological function or activity as such polypeptide. Thus, an analog includes a proprotein which can be activated by cleavage of the proprotein portion to produce an active mature polypeptide.

The polypeptide of the present invention may be a recombinant polypeptide, a natural polypeptide or a synthetic polypeptide, preferably a recombinant polypeptide.

The fragment, derivative or analog of the polypeptide of Figure 1 (SEQ ID NO:2) or that encoded by the deposited cDNA may be (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code, or (ii) one in which one or more of the amino acid residues includes a substituent group, or (iii) one in which the mature polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene

glycol), or (iv) one in which the additional amino acids are fused to the mature polypeptide, such as a leader or secretory sequence or a sequence which is employed for purification of the mature polypeptide or a proprotein sequence. Such fragments, derivatives and analogs are deemed to be within the scope of those skilled in the art from the teachings herein.

The polypeptides and polynucleotides of the present invention are preferably provided in an isolated form, and preferably are purified to homogeneity.

The term "isolated" means that the material is removed from its original environment (e.g., the natural environment if it is naturally occurring). For example, a naturally-occurring polynucleotide or polypeptide present in a living animal is not isolated, but the same polynucleotide or polypeptide, separated from some or all of the coexisting materials in the natural system, is isolated. Such polynucleotides could be part of a vector and/or such polynucleotides or polypeptides could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment.

The polypeptides of the present invention include the polypeptide of SEQ ID NO:2 (in particular the mature polypeptide) as well as polypeptides which have at least 70% similarity (preferably a 70% identity) to the polypeptide of SEQ ID NO:2 and more preferably a 90% similarity (more preferably a 90% identity) to the polypeptide of SEQ ID NO:2 and still more preferably a 95% similarity (still more preferably a 90% identity) to the polypeptide of SEQ ID NO:2 and also include portions of such polypeptides with such portion of the polypeptide generally containing at least 30 amino acids and more preferably at least 50 amino acids.

As known in the art "similarity" between two polypeptides is determined by comparing the amino acid

sequence and its conserved amino acid substitutes of one polypeptide to the sequence of a second polypeptide.

Fragments or portions of the polypeptides of the present invention may be employed for producing the corresponding full-length polypeptide by peptide synthesis; therefore, the fragments may be employed as intermediates for producing the full-length polypeptides. Fragments or portions of the polynucleotides of the present invention may be used to synthesize full-length polynucleotides of the present invention.

The present invention also relates to vectors which include polynucleotides of the present invention, host cells which are genetically engineered with vectors of the invention and the production of polypeptides of the invention by recombinant techniques.

Host cells are genetically engineered (transduced or transformed or transfected) with the vectors of this invention which may be, for example, a cloning vector or an expression vector. The vector may be, for example, in the form of a plasmid, a viral particle, a phage, etc. The engineered host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants or amplifying the genes of the present invention. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

The polynucleotides of the present invention may be employed for producing polypeptides by recombinant techniques. Thus, for example, the polynucleotide may be included in any one of a variety of expression vectors for expressing a polypeptide. Such vectors include chromosomal, nonchromosomal and synthetic DNA sequences, e.g., derivatives of SV40; bacterial plasmids; phage DNA; baculovirus; yeast plasmids; vectors derived from combinations of plasmids and

phage DNA, viral DNA such as vaccinia, adenovirus, fowl pox virus, and pseudorabies. However, any other vector may be used as long as it is replicable and viable in the host.

The appropriate DNA sequence may be inserted into the vector by a variety of procedures. In general, the DNA sequence is inserted into an appropriate restriction endonuclease site(s) by procedures known in the art. Such procedures and others are deemed to be within the scope of those skilled in the art.

The DNA sequence in the expression vector is operatively linked to an appropriate expression control sequence(s) (promoter) to direct mRNA synthesis. As representative examples of such promoters, there may be mentioned: LTR or SV40 promoter, the E. coli. lac or trp, the phage lambda P<sub>L</sub> promoter and other promoters known to control expression of genes in prokaryotic or eukaryotic cells or their viruses. The expression vector also contains a ribosome binding site for translation initiation and a transcription terminator. The vector may also include appropriate sequences for amplifying expression.

In addition, the expression vectors preferably contain one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells such as dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, or such as tetracycline or ampicillin resistance in E. coli.

The vector containing the appropriate DNA sequence as hereinabove described, as well as an appropriate promoter or control sequence, may be employed to transform an appropriate host to permit the host to express the protein.

As representative examples of appropriate hosts, there may be mentioned: bacterial cells, such as E. coli, Streptomyces, Salmonella typhimurium; fungal cells, such as yeast; insect cells such as Drosophila S2 and Spodoptera Sf9; animal cells such as CHO, COS or Bowes melanoma;



adenoviruses; plant cells, etc. The selection of an appropriate host is deemed to be within the scope of those skilled in the art from the teachings herein.

More particularly, the present invention also includes recombinant constructs comprising one or more of the sequences as broadly described above. The constructs comprise a vector, such as a plasmid or viral vector, into which a sequence of the invention has been inserted, in a forward or reverse orientation. In a preferred aspect of this embodiment, the construct further comprises regulatory sequences, including, for example, a promoter, operably linked to the sequence. Large numbers of suitable vectors and promoters are known to those of skill in the art, and are commercially available. The following vectors are provided by way of example; Bacterial: pQE70, pQE60, pQE-9 (Qiagen), pBS, pD10, phagescript, psiX174, pbluescript SK, pbsks, pNH8A, pNH16a, pNH18A, pNH46A (Stratagene); ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 (Pharmacia); Eukaryotic: pWLNEO, pSV2CAT, pOG44, pXT1, pSG (Stratagene) pSVK3, pBPV, pMSG, pSVL (Pharmacia). However, any other plasmid or vector may be used as long as they are replicable and viable in the host.

Promoter regions can be selected from any desired gene using CAT (chloramphenicol transferase) vectors or other vectors with selectable markers. Two appropriate vectors are pKK232-8 and pCM7. Particular named bacterial promoters include lacI, lacZ, T3, T7, gpt, lambda P<sub>R</sub>, P<sub>L</sub> and trp. Eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retrovirus, and mouse metallothionein-I. Selection of the appropriate vector and promoter is well within the level of ordinary skill in the art.

In a further embodiment, the present invention relates to host cells containing the above-described constructs. The host cell can be a higher eukaryotic cell, such as a

mammalian cell, or a lower eukaryotic cell, such as a yeast cell, or the host cell can be a prokaryotic cell, such as a bacterial cell. Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-Dextran mediated transfection, or electroporation (Davis, L., Dibner, M., Battey, I., Basic Methods in Molecular Biology, (1986)).

The constructs in host cells can be used in a conventional manner to produce the gene product encoded by the recombinant sequence. Alternatively, the polypeptides of the invention can be synthetically produced by conventional peptide synthesizers.

Mature proteins can be expressed in mammalian cells, yeast, bacteria, or other cells under the control of appropriate promoters. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention. Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described by Sambrook, et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, N.Y., (1989), the disclosure of which is hereby incorporated by reference.

Transcription of the DNA encoding the polypeptides of the present invention by higher eukaryotes is increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp that act on a promoter to increase its transcription. Examples including the SV40 enhancer on the late side of the replication origin bp 100 to 270, a cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

Generally, recombinant expression vectors will include origins of replication and selectable markers permitting transformation of the host cell, e.g., the ampicillin resistance gene of E. coli and S. cerevisiae TRP1 gene, and

a promoter derived from a highly-expressed gene to direct transcription of a downstream structural sequence. Such promoters can be derived from operons encoding glycolytic enzymes such as 3-phosphoglycerate kinase (PGK),  $\alpha$ -factor, acid phosphatase, or heat shock proteins, among others. The heterologous structural sequence is assembled in appropriate phase with translation initiation and termination sequences, and preferably, a leader sequence capable of directing secretion of translated protein into the periplasmic space or extracellular medium. Optionally, the heterologous sequence can encode a fusion protein including an N-terminal identification peptide imparting desired characteristics, e.g., stabilization or simplified purification of expressed recombinant product.

Useful expression vectors for bacterial use are constructed by inserting a structural DNA sequence encoding a desired protein together with suitable translation initiation and termination signals in operable reading phase with a functional promoter. The vector will comprise one or more phenotypic selectable markers and an origin of replication to ensure maintenance of the vector and to, if desirable, provide amplification within the host. Suitable prokaryotic hosts for transformation include E. coli, Bacillus subtilis, Salmonella typhimurium and various species within the genera Pseudomonas, Streptomyces, and Staphylococcus, although others may also be employed as a matter of choice.

As a representative but nonlimiting example, useful expression vectors for bacterial use can comprise a selectable marker and bacterial origin of replication derived from commercially available plasmids comprising genetic elements of the well known cloning vector pBR322 (ATCC 37017). Such commercial vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and GEM1 (Promega Biotec, Madison, WI, USA). These pBR322 "backbone"

sections are combined with an appropriate promoter and the structural sequence to be expressed.

Following transformation of a suitable host strain and growth of the host strain to an appropriate cell density, the selected promoter is induced by appropriate means (e.g., temperature shift or chemical induction) and cells are cultured for an additional period.

Cells are typically harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract retained for further purification.

Microbial cells employed in expression of proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents, such methods are well known to those skilled in the art.

Various mammalian cell culture systems can also be employed to express recombinant protein. Examples of mammalian expression systems include the COS-7 lines of monkey kidney fibroblasts, described by Gluzman, Cell, 23:175 (1981), and other cell lines capable of expressing a compatible vector, for example, the C127, 3T3, CHO, HeLa and BHK cell lines. Mammalian expression vectors will comprise an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking nontranscribed sequences. DNA sequences derived from the SV40 splice, and polyadenylation sites may be used to provide the required nontranscribed genetic elements.

The polypeptides can be recovered and purified from recombinant cell cultures by methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and

lectin chromatography. Protein refolding steps can be used, as necessary, in completing configuration of the mature protein. Finally, high performance liquid chromatography (HPLC) can be employed for final purification steps.

The polypeptides of the present invention may be a naturally purified product, or a product of chemical synthetic procedures, or produced by recombinant techniques from a prokaryotic or eukaryotic host (for example, by bacterial, yeast, higher plant, insect and mammalian cells in culture). Depending upon the host employed in a recombinant production procedure, the polypeptides of the present invention may be glycosylated or may be non-glycosylated. Polypeptides of the invention may also include an initial methionine amino acid residue.

The polynucleotides and polypeptides of the present invention may be employed as research reagents and materials for discovery of treatments and diagnostics for human disease.

The polypeptide of the present invention may be employed for characterization of receptors. The EGF family receptors currently includes four EGF receptors, denoted as EGFR1, EGFR2, EGFR3 and EGFR4. The EGFR2 receptor may also be referred to as ERB-2 and this molecule is useful for a variety of diagnostic and therapeutic indications (Prigent, S.A., and Lemoine, N.R., Prog Growth Factor Res., 4:1-24 (1992)). The TGF $\alpha$ -HII polypeptide is likely a ligand for one or more of these receptors as well as for yet an identified new EGF-type receptor. Use of the TGF $\alpha$ -HII can assist with the identification, characterization and cloning of such receptors. For example, the EGF receptor gene represents the cellular homolog of the v-erb-B oncogene of avian erythroblastosis virus. Over expression of the EGF-receptor or deletion of kinase regulatory segments of the protein can bring about tumorigenic transformation of cells (Manjusri, D. et al., Human Cytokines, 364 and 381 (1991)).

The polypeptides of the present invention may also be employed for restoration or enhancement of neurological functions diminished as a result of trauma or other damaging pathologies (such as AIDS dementia, senile dementia, etc). TGF $\alpha$  and its homologs have been found to be the most abundant ligand for the EGF/TGF $\alpha$  receptor in most parts of the brain (Kaser, et al., Brain Res Mol Brain Res: 16:316-322, (1992)). There appears to be a widespread distribution of TGF $\alpha$  in various regions of the brain in contrast to EGF which is only present in smaller, more discrete areas, suggesting that TGF-alpha might play a physiological role in brain tissues. These numerous receptor sites for TGF $\alpha$  in the brain suggest that TGF has an important utility in promoting normal brain cell differentiation and function. Accordingly, in instances where neurological functioning is diminished, an administration of the polypeptide of the present invention may stimulate the brain and enhance proper physiological functions.

TGF $\alpha$ -HII or soluble form thereof may also be employed to treat ocular disorders, for example, corneal inflammation. A variety of experiments have implicated members of the TGF $\alpha$  gene family in such pathologies. A recent paper summarizes some of the data related to the role these growth factors play in eye disease (Mann et al Cell 73:249-261 (1993)). Recent experiments have shown that a number of mice lacking the TGF $\alpha$  gene displayed corneal inflammation due to an infiltration of leukocytes and other cells to the substantia propria of the eyes.

In addition, the specificity of the TGF $\alpha$  growth factors for their target cells can be exploited as a mechanism to destroy the target cell. For example, TGF $\alpha$ -HII or soluble forms thereof can be coupled (by a wide variety of methods) to toxic molecules: for example, a radiopharmaceutical which inactivate target cells. These growth factor-toxin fusions kill the target cell (and in certain cases neighboring cells

by a variety of "bystander" effects). A recent example of such toxin-fusion genes is published by Mesri, et al., J. Biol. Chem. 268:4853-62 (1993). TGF $\alpha$ -HII and related molecules may also be encapsulated in liposomes and may be conjugated to antibodies which recognize and bind to tumor or cell specific antigens, thereby provided a means for "targeting" cells.

In this same manner, TGF $\alpha$ -HII can be employed as an anti-neoplastic compound, since members of the EGF family show anti-proliferative effects on transformed cells. For in vivo use, the subject polypeptide may be administered in a variety of ways, including but not limited to, injection, infusion, topically, parenterally, etc. Administration may be in any physiologically acceptable carrier, including phosphate buffered saline, saline, sterilized water, etc.

The TGF $\alpha$ -HII polypeptide fragment may also be employed to treat certain kidney disorders, since it has been found that there has been expression of these growth factors in the kidney. Thus, these factors may be necessary for the proper physiological maintenance of this organ.

Treatments may also be related to liver regeneration or liver dysfunction, since TGF $\alpha$  and its homologs and hepatocyte growth factor trigger hepatocyte regeneration after partial hepatectomy and after acute liver cell necrosis (Masuhara, M. et al, Hepatology 16:1241-1249 (1992)).

A significant treatment involving TGF $\alpha$ -HII relates to wound healing. The compositions of the present invention may be employed for treating a wide variety of wounds including substantially all cutaneous wounds, corneal wounds, and injuries to the epithelial-lined hollow organs of the body. Wounds suitable for treatment include those resulting from trauma such as burns, abrasions and cuts, as well as from surgical procedures such as surgical incisions and skin grafting. Other conditions suitable for treatment with the polypeptide of the present invention include chronic

conditions, such as chronic ulcers, diabetic ulcers, and other non-healing (trophic) conditions.

TGF $\alpha$ -HII or soluble fragment thereof may be incorporated in physiologically-acceptable carriers for application to the affected area. The nature of the carriers may vary widely and will depend on the intended location of application. For application to the skin, a cream or ointment base is usually preferred; suitable bases include lanolin, Silvadene (Marion) (particularly for the treatment of burns), Aquaphor (Duke Laboratories, South Norwalk, Conn.), and the like. If desired, it will be possible to incorporate TGF $\alpha$ -HII containing compositions in bandages and other wound dressings to provide for continuous exposure of the wound to the peptide. Aerosol applications may also find use.

The concentration of TGF $\alpha$ -HII in the treatment composition is not critical but should be enough to induce epithelial cell proliferation. The compositions may be applied topically to the affected area, typically as eye drops to the eye or as creams, ointments or lotions to the skin. In the case of the eyes, frequent treatment is desirable, usually being applied at intervals of 4 hours or less. On the skin, it is desirable to continually maintain the treatment composition on the affected area during the healing, with applications of the treatment composition from two to four times a day or more frequently.

The amount employed of the subject polypeptide will vary with the manner of administration, the employment of other active compounds, and the like, generally being in the range of about 1  $\mu$ g to 100  $\mu$ g. The subject polypeptide may be employed with a physiologically acceptable carrier, such as saline, phosphate-buffered saline, or the like. The amount of compound employed will be determined empirically, based on the response of cells in vitro and response of experimental animals to the subject polypeptides or formulations containing the subject polypeptides.



The TGF $\alpha$ -HII or soluble fragment thereof may be employed in the modulation of angiogenesis, bone resorption, immune response, and synaptic and neuronal effector functions. TGF $\alpha$ -HII may also be used in the modulation of the arachidonic acid cascade.

TGF $\alpha$ -HII or soluble fragment thereof may also be employed for applications related to terminal differentiation. Many TGF $\alpha$  factors, and their homologs, induce terminal differentiation in their target cells. This property can be exploited in vivo by administering the factor and inducing target cell death. This regimen is under consideration for disorders related to the hyperproliferation of medically undesirable cell types such as cancers and other proliferative disorders (eg inflammation, psoriasis, etc). In addition to in vivo administration, there are a variety of situations where in vitro administration may be warranted. For example, bone marrow can be purged of undesirable cell populations in vitro by treating the cells with growth factors and/or derivatives thereof.

Applications are also related to alopecia, hair loss and to other skin conditions which affect hair follicular development. Several lines of evidence implicate the involvement TGF $\alpha$  growth factors in such conditions. As described above, "knockout" mice engineered to contain a null mutation in the TGF $\alpha$  gene display abnormalities related to quantitative and qualitative hair synthesis. In addition, mapping studies in mice have shown that some mutations affecting hair growth map to the TGF $\alpha$  gene locus (Mann et al, Cell 73:249-261(1993)). Topical or systemic applications of TGF $\alpha$ -HII or derivatives thereof may be employed to treat some forms of alopecia and hair loss and these claims fall within the scope of this invention.

Certain disease pathologies may be partially or completely ameliorated by the systemic clinical

administration of the TGF $\alpha$ -HII growth factor. This administration can be in the form of gene therapy (see below); or through the administration of peptides or proteins synthesized from recombinant constructs of TGF $\alpha$ -HII DNA or from peptide chemical synthesis (Woo, et al., Protein Engineering 3:29-37 (1989)).

This invention provides a method of screening compounds to identify agonist or antagonist compounds to the polypeptide of the present invention. As an example, a mammalian cell or membrane preparation expressing a TGF $\alpha$ -HII receptor is incubated with a potential compound and the ability of the compound to generate a second signal from the receptor is measured to determine if it is an effective agonist. Such second messenger systems include but are not limited to, cAMP guanylate cyclase, ion channels or phosphoinositide hydrolysis. Effective antagonists are determined by the method above wherein an antagonist compound is detected which binds to the receptor but does not elicit a second messenger response to thereby block the receptor from TGF $\alpha$ -HII.

Another assay for identifying potential antagonists specific to the receptors to the polypeptide of the present invention is a competition assay which comprises isolating plasma membranes which over express a receptor to the polypeptide of the present invention, for example, human A431 carcinoma cells. Serially diluted test sample in a medium (volume is approximately 10 microliters) containing 10 nM <sup>125</sup>I-TGF $\alpha$ -HII is added to five micrograms of the plasma membrane in the presence of the potential antagonist compound and incubated for 4 hours at 4°C. The reaction mixtures are diluted and immediately passed through a millipore filter. The filters are then rapidly washed and the bound radioactivity is measured in a gamma counter. The amount of bound TGF $\alpha$ -HII is then measured. A control assay is also

performed in the absence of the compound to determine if the antagonists reduce the amount of bound TGF $\alpha$ -HII.

Potential antagonist compounds include an antibody, or in some cases, an oligopeptide, which binds to the polypeptide. Alternatively, a potential antagonist may be a closely related protein which binds to the receptor which is an inactive forms of the polypeptide and thereby prevent the action of the polypeptide of the present invention.

Another antagonist compound is an antisense construct prepared using antisense technology. Antisense technology can be used to control gene expression through triple-helix formation or antisense DNA or RNA, both of which methods are based on binding of a polynucleotide to DNA or RNA. For example, the 5' coding portion of the polynucleotide sequence, which encodes for the mature polypeptides of the present invention, is used to design an antisense RNA oligonucleotide of from about 10 to 40 base pairs in length. A DNA oligonucleotide is designed to be complementary to a region of the gene involved in transcription (triple helix - see Lee et al., Nucl. Acids Res., 6:3073 (1979); Cooney et al, Science, 241:456 (1988); and Dervan et al., Science, 251: 1360 (1991)), thereby preventing transcription and the production of the polypeptide of the present invention. The antisense RNA oligonucleotide hybridizes to the mRNA *in vivo* and blocks translation of the mRNA molecule into the polypeptide of the present invention (Antisense - Okano, J. Neurochem., 56:560 (1991); Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression, CRC Press, Boca Raton, FL (1988)). The oligonucleotides described above can also be delivered to cells such that the antisense RNA or DNA may be expressed *in vivo* to inhibit production of the polypeptide of the present invention.

Antagonist compounds include a small molecule which binds to the polypeptide of the present invention and blocks its action at the receptor such that normal biological

activity is prevented. The small molecules may also bind the receptor to the polypeptide to prevent binding. Examples of small molecules include but are not limited to small peptides or peptide-like molecules.

The antagonists may be employed to treat neoplasia, for example, cancers and tumors. It is known that inhibition of secretion or production of members of the EGF family by tumor cells in mice causes regression of tumors.

The antagonists to the polypeptides of the present invention may also be used therapeutically for the treatment of certain skin disorders, for example, psoriasis. Elevated levels of expression of members of this family of growth factors in skin biopsies taken from diseases such as psoriatic lesions have been found to be elevated (Cook, et al., Cancer Research, 52:3224-3227 (1992)). The antagonists may be employed in a composition with a pharmaceutically acceptable carrier, e.g., as hereinafter described.

The polypeptides of the present invention or agonist or antagonist compounds may be employed in combination with a suitable pharmaceutical carrier. Such compositions comprise a therapeutically effective amount of the polypeptide or compound, and a pharmaceutically acceptable carrier or excipient. Such a carrier includes but is not limited to saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. The formulation should suit the mode of administration.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration. In addition, the polypeptides or compounds of the present

invention may be employed in conjunction with other therapeutic compounds.

The pharmaceutical compositions may be administered in a convenient manner such as by the oral, topical, intravenous, intraperitoneal, intramuscular, subcutaneous, intranasal or intradermal routes. The pharmaceutical compositions are administered in an amount which is effective for treating and/or prophylaxis of the specific indication. In general, they are administered in an amount of at least about 10  $\mu\text{g/kg}$  body weight and in most cases they will be administered in an amount not in excess of about 8 mg/Kg body weight per day. In most cases, the dosage is from about 10  $\mu\text{g/kg}$  to about 1 mg/kg body weight daily, taking into account the routes of administration, symptoms, etc.

The polypeptides, and agonists and antagonists which are polypeptides, may also be employed in accordance with the present invention by expression of such polypeptides *in vivo*, which is often referred to as "gene therapy."

Thus, for example, cells from a patient may be engineered with a polynucleotide (DNA or RNA) encoding a polypeptide *ex vivo*, with the engineered cells then being provided to a patient to be treated with the polypeptide. Such methods are well-known in the art and are apparent from the teachings herein. For example, cells may be engineered by the use of a retroviral plasmid vector containing RNA encoding a polypeptide of the present invention.

Similarly, cells may be engineered *in vivo* for expression of a polypeptide *in vivo* by, for example, procedures known in the art. For example, a packaging cell is transduced with a retroviral plasmid vector containing RNA encoding a polypeptide of the present invention such that the packaging cell now produces infectious viral particles containing the gene of interest. These producer cells may be administered to a patient for engineering cells *in vivo* and expression of the polypeptide *in vivo*. These and other

methods for administering a polypeptide of the present invention by such method should be apparent to those skilled in the art from the teachings of the present invention.

Retroviruses from which the retroviral plasmid vectors hereinabove mentioned may be derived include, but are not limited to, Moloney Murine Leukemia Virus, spleen necrosis virus, retroviruses such as Rous Sarcoma Virus, Harvey Sarcoma Virus, avian leukosis virus, gibbon ape leukemia virus, human immunodeficiency virus, adenovirus, Myeloproliferative Sarcoma Virus, and mammary tumor virus. In one embodiment, the retroviral plasmid vector is derived from Moloney Murine Leukemia Virus.

The vector includes one or more promoters. Suitable promoters which may be employed include, but are not limited to, the retroviral LTR; the SV40 promoter; and the human cytomegalovirus (CMV) promoter described in Miller, et al., Biotechniques, Vol. 7, No. 9, 980-990 (1989), or any other promoter (e.g., cellular promoters such as eukaryotic cellular promoters including, but not limited to, the histone, pol III, and  $\beta$ -actin promoters). Other viral promoters which may be employed include, but are not limited to, adenovirus promoters, thymidine kinase (TK) promoters, and B19 parvovirus promoters. The selection of a suitable promoter will be apparent to those skilled in the art from the teachings contained herein.

The nucleic acid sequence encoding the polypeptide of the present invention is under the control of a suitable promoter. Suitable promoters which may be employed include, but are not limited to, adenoviral promoters, such as the adenoviral major late promoter; or heterologous promoters, such as the cytomegalovirus (CMV) promoter; the respiratory syncytial virus (RSV) promoter; inducible promoters, such as the MMT promoter, the metallothionein promoter; heat shock promoters; the albumin promoter; the ApoAI promoter; human globin promoters; viral thymidine kinase promoters, such as

the Herpes Simplex thymidine kinase promoter; retroviral LTRs (including the modified retroviral LTRs hereinabove described); the  $\beta$ -actin promoter; and human growth hormone promoters. The promoter also may be the native promoter which controls the gene encoding the polypeptide.

The retroviral plasmid vector is employed to transduce packaging cell lines to form producer cell lines. Examples of packaging cells which may be transfected include, but are not limited to, the PE501, PA317,  $\psi$ -2,  $\psi$ -AM, PA12, T19-14X, VT-19-17-H2,  $\psi$ CRE,  $\psi$ CRIP, GP+E-86, GP+envAm12, and DAN cell lines as described in Miller, Human Gene Therapy, Vol. 1, pgs. 5-14 (1990), which is incorporated herein by reference in its entirety. The vector may transduce the packaging cells through any means known in the art. Such means include, but are not limited to, electroporation, the use of liposomes, and  $\text{CaPO}_4$  precipitation. In one alternative, the retroviral plasmid vector may be encapsulated into a liposome, or coupled to a lipid, and then administered to a host.

The producer cell line generates infectious retroviral vector particles which include the nucleic acid sequence(s) encoding the polypeptides. Such retroviral vector particles then may be employed, to transduce eukaryotic cells, either *in vitro* or *in vivo*. The transduced eukaryotic cells will express the nucleic acid sequence(s) encoding the polypeptide. Eukaryotic cells which may be transduced include, but are not limited to, embryonic stem cells, embryonic carcinoma cells, as well as hematopoietic stem cells, hepatocytes, fibroblasts, myoblasts, keratinocytes, endothelial cells, and bronchial epithelial cells.

This invention is also related to the use of the gene of the present invention as a diagnostic. Detection of a mutated form of the gene of the present invention will allow a diagnosis of a disease or a susceptibility to a disease which results from underexpression of the polypeptide of the

present invention for example, improper wound healing, improper neurological functioning, ocular disorders, kidney and liver disorders, hair follicular development, angiogenesis and embryogenesis.

Individuals carrying mutations in the human gene of the present invention may be detected at the DNA level by a variety of techniques. Nucleic acids for diagnosis may be obtained from a patient's cells, such as from blood, urine, saliva, tissue biopsy and autopsy material. The genomic DNA may be used directly for detection or may be amplified enzymatically by using PCR (Saiki et al., Nature, 324:163-166 (1986)) prior to analysis. RNA or cDNA may also be used for the same purpose. As an example, PCR primers complementary to the nucleic acid encoding a polypeptide of the present invention can be used to identify and analyze mutations thereof. For example, deletions and insertions can be detected by a change in size of the amplified product in comparison to the normal genotype. Point mutations can be identified by hybridizing amplified DNA to radiolabeled RNA or alternatively, radiolabeled antisense DNA sequences. Perfectly matched sequences can be distinguished from mismatched duplexes by RNase A digestion or by differences in melting temperatures.

Sequence differences between the reference gene and genes having mutations may be revealed by the direct DNA sequencing method. In addition, cloned DNA segments may be employed as probes to detect specific DNA segments. The sensitivity of this method is greatly enhanced when combined with PCR. For example, a sequencing primer is used with double-stranded PCR product or a single-stranded template molecule generated by a modified PCR. The sequence determination is performed by conventional procedures with radiolabeled nucleotide or by automatic sequencing procedures with fluorescent-tags.



Genetic testing based on DNA sequence differences may be achieved by detection of alteration in electrophoretic mobility of DNA fragments in gels with or without denaturing agents. Small sequence deletions and insertions can be visualized by high resolution gel electrophoresis. DNA fragments of different sequences may be distinguished on denaturing formamide gradient gels in which the mobilities of different DNA fragments are retarded in the gel at different positions according to their specific melting or partial melting temperatures (see, e.g., Myers et al., Science, 230:1242 (1985)).

Sequence changes at specific locations may also be revealed by nuclease protection assays, such as RNase and S1 protection or the chemical cleavage method (e.g., Cotton et al., PNAS, USA, 85:4397-4401 (1985)).

Thus, the detection of a specific DNA sequence may be achieved by methods such as hybridization, RNase protection, chemical cleavage, direct DNA sequencing or the use of restriction enzymes, (e.g., Restriction Fragment Length Polymorphisms (RFLP)) and Southern blotting of genomic DNA.

In addition to more conventional gel-electrophoresis and DNA sequencing, mutations can also be detected by *in situ* analysis.

The present invention also relates to diagnostic assays for detecting altered levels of the polypeptide of the present invention in various tissues since an over-expression of the proteins compared to normal control tissue samples can detect the presence of certain disease conditions such as neoplasia, skin disorders, ocular disorders and inflammation. Assays used to detect levels of the polypeptide of the present invention in a sample derived from a host are well-known to those of skill in the art and include radioimmunoassays, competitive-binding assays, Western Blot analysis and preferably an ELISA assay. An ELISA assay initially comprises preparing an antibody specific to an

antigen of the polypeptide of the present invention, preferably a monoclonal antibody. In addition a reporter antibody is prepared against the monoclonal antibody. To the reporter antibody is attached a detectable reagent such as radioactivity, fluorescence or in this example a horseradish peroxidase enzyme. A sample is now removed from a host and incubated on a solid support, e.g. a polystyrene dish, that binds the proteins in the sample. Any free protein binding sites on the dish are then covered by incubating with a non-specific protein such as bovine serum albumin. Next, the monoclonal antibody is incubated in the dish during which time the monoclonal antibodies attach to any polypeptides of the present invention attached to the polystyrene dish. All unbound monoclonal antibody is washed out with buffer. The reporter antibody linked to horseradish peroxidase is now placed in the dish resulting in binding of the reporter antibody to any monoclonal antibody bound to polypeptides of the present invention. Unattached reporter antibody is then washed out. Peroxidase substrates are then added to the dish and the amount of color developed in a given time period is a measurement of the amount of protein present in a given volume of patient sample when compared against a standard curve.

A competition assay may also be employed to determine levels of the polypeptide of the present invention in a sample derived from the hosts. Such an assay comprises isolating plasma membranes which over-express the receptor for the polypeptide of the present invention. A test sample containing the polypeptides of the present invention which have been labeled, are then added to the plasma membranes and then incubated for a set period of time. Also added to the reaction mixture is a sample derived from a host which is suspected of containing the polypeptide of the present invention. The reaction mixtures are then passed through a filter which is rapidly washed and the bound radioactivity is

then measured to determine the amount of competition for the receptors and therefore the amount of the polypeptides of the present invention in the sample.

Antibodies specific to TGF $\alpha$ -HII may be used for cancer diagnosis and therapy, since many types of cancer cells up-regulate various members of the TGF $\alpha$  family during the process of neoplasia or hyperplasia. These antibodies bind to and inactivate TGF $\alpha$ -HII. Monoclonal antibodies against TGF $\alpha$ -HII (and/or its family members) are in clinical use for both the diagnosis and therapy of certain disorders including (but not limited to) hyperplastic and neoplastic growth abnormalities. Upregulation of growth factor expression by neoplastic tissues forms the basis for a variety of serum assays which detect increases in growth factor in the blood of affected patients. These assays are typically applied not only in diagnostic settings, but are applied in prognostic settings as well (to detect the presence of occult tumor cells following surgery, chemotherapy, etc).

In addition, malignant cells expressing the TGF $\alpha$ -HII receptor may be detected by using labeled TGF $\alpha$ -HII in a receptor binding assay, or by the use of antibodies to the TGF $\alpha$ -HII receptor itself. Cells may be distinguished in accordance with the presence and density of receptors for TGF $\alpha$ -HII, thereby providing a means for predicting the susceptibility of such cells to the biological activities of TGF $\alpha$ -HII.

The sequences of the present invention are also valuable for chromosome identification. The sequence is specifically targeted to and can hybridize with a particular location on an individual human chromosome. Moreover, there is a current need for identifying particular sites on the chromosome. Few chromosome marking reagents based on actual sequence data (repeat polymorphisms) are presently available for marking chromosomal location. The mapping of DNAs to chromosomes according to the present invention is an important first step

in correlating those sequences with genes associated with disease.

Briefly, sequences can be mapped to chromosomes by preparing PCR primers (preferably 15-25 bp) from the cDNA. Computer analysis of the 3' untranslated region of the gene is used to rapidly select primers that do not span more than one exon in the genomic DNA, thus complicating the amplification process. These primers are then used for PCR screening of somatic cell hybrids containing individual human chromosomes. Only those hybrids containing the human gene corresponding to the primer will yield an amplified fragment.

PCR mapping of somatic cell hybrids is a rapid procedure for assigning a particular DNA to a particular chromosome. Using the present invention with the same oligonucleotide primers, sublocalization can be achieved with panels of fragments from specific chromosomes or pools of large genomic clones in an analogous manner. Other mapping strategies that can similarly be used to map to its chromosome include *in situ* hybridization, prescreening with labeled flow-sorted chromosomes and preselection by hybridization to construct chromosome specific-cDNA libraries.

Fluorescence *in situ* hybridization (FISH) of a cDNA clone to a metaphase chromosomal spread can be used to provide a precise chromosomal location in one step. This technique can be used with cDNA as short as 50 or 60 bases. For a review of this technique, see Verma et al., *Human Chromosomes: a Manual of Basic Techniques*, Pergamon Press, New York (1988).

Once a sequence has been mapped to a precise chromosomal location, the physical position of the sequence on the chromosome can be correlated with genetic map data. Such data are found, for example, in V. McKusick, *Mendelian Inheritance in Man* (available on line through Johns Hopkins University Welch Medical Library). The relationship between genes and diseases that have been mapped to the same

chromosomal region are then identified through linkage analysis (coinheritance of physically adjacent genes).

Next, it is necessary to determine the differences in the cDNA or genomic sequence between affected and unaffected individuals. If a mutation is observed in some or all of the affected individuals but not in any normal individuals, then the mutation is likely to be the causative agent of the disease.

With current resolution of physical mapping and genetic mapping techniques, a cDNA precisely localized to a chromosomal region associated with the disease could be one of between 50 and 500 potential causative genes. (This assumes 1 megabase mapping resolution and one gene per 20 kb).

The polypeptides, their fragments or other derivatives, or analogs thereof, or cells expressing them can be used as an immunogen to produce antibodies thereto. These antibodies can be, for example, polyclonal or monoclonal antibodies. The present invention also includes chimeric, single chain, and humanized antibodies, as well as Fab fragments, or the product of an Fab expression library. Various procedures known in the art may be used for the production of such antibodies and fragments.

Antibodies generated against the polypeptides corresponding to a sequence of the present invention can be obtained by direct injection of the polypeptides into an animal or by administering the polypeptides to an animal, preferably a nonhuman. The antibody so obtained will then bind the polypeptides itself. In this manner, even a sequence encoding only a fragment of the polypeptides can be used to generate antibodies binding the whole native polypeptides. Such antibodies can then be used to isolate the polypeptide from tissue expressing that polypeptide.

For preparation of monoclonal antibodies, any technique which provides antibodies produced by continuous cell line

cultures can be used. Examples include the hybridoma technique (Kohler and Milstein, 1975, Nature, 256:495-497), the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983, Immunology Today 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole, et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96).

Techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce single chain antibodies to immunogenic polypeptide products of this invention. Also, transgenic mice may be used to express humanized antibodies to immunogenic polypeptide products of this invention.

The present invention will be further described with reference to the following examples; however, it is to be understood that the present invention is not limited to such examples. All parts or amounts, unless otherwise specified, are by weight.

In order to facilitate understanding of the following examples certain frequently occurring methods and/or terms will be described.

"Plasmids" are designated by a lower case p preceded and/or followed by capital letters and/or numbers. The starting plasmids herein are either commercially available, publicly available on an unrestricted basis, or can be constructed from available plasmids in accord with published procedures. In addition, equivalent plasmids to those described are known in the art and will be apparent to the ordinarily skilled artisan.

"Digestion" of DNA refers to catalytic cleavage of the DNA with a restriction enzyme that acts only at certain sequences in the DNA. The various restriction enzymes used herein are commercially available and their reaction conditions, cofactors and other requirements were used as would be known to the ordinarily skilled artisan. For

analytical purposes, typically 1  $\mu$ g of plasmid or DNA fragment is used with about 2 units of enzyme in about 20  $\mu$ l of buffer solution. For the purpose of isolating DNA fragments for plasmid construction, typically 5 to 50  $\mu$ g of DNA are digested with 20 to 250 units of enzyme in a larger volume. Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer. Incubation times of about 1 hour at 37°C are ordinarily used, but may vary in accordance with the supplier's instructions. After digestion the reaction is electrophoresed directly on a polyacrylamide gel to isolate the desired fragment.

Size separation of the cleaved fragments is performed using 8 percent polyacrylamide gel described by Goeddel, D. et al., *Nucleic Acids Res.*, 8:4057 (1980).

"Oligonucleotides" refers to either a single stranded polydeoxynucleotide or two complementary polydeoxynucleotide strands which may be chemically synthesized. Such synthetic oligonucleotides have no 5' phosphate and thus will not ligate to another oligonucleotide without adding a phosphate with an ATP in the presence of a kinase. A synthetic oligonucleotide will ligate to a fragment that has not been dephosphorylated.

"Ligation" refers to the process of forming phosphodiester bonds between two double stranded nucleic acid fragments (Maniatis, T., et al., *Id.*, p. 146). Unless otherwise provided, ligation may be accomplished using known buffers and conditions with 10 units of T4 DNA ligase ("ligase") per 0.5  $\mu$ g of approximately equimolar amounts of the DNA fragments to be ligated.

Unless otherwise stated, transformation was performed as described in the method of Graham, F. and Van der Eb, A., *Virology*, 52:456-457 (1973).

Example 1Bacterial Expression and Purification of the soluble form of TGF $\alpha$ -HII

The DNA sequence encoding TGF $\alpha$ -HII, ATCC # \_\_\_\_\_, was initially amplified using PCR oligonucleotide primers corresponding to the 5' sequences of the processed TGF $\alpha$ -HII protein (minus the signal peptide sequence) and the vector sequences 3' to the TGF $\alpha$ -HII gene. Additional nucleotides corresponding to TGF $\alpha$ -HII were added to the 5' and 3' sequences respectively. The 5' oligonucleotide primer has the sequence 5' CCCGGATCCGCACGAGACATACCTTGTCCG 3' (SEQ ID NO:3) contains a BamHI restriction enzyme site (in bold) followed by 21 nucleotides of TGF $\alpha$ -HII coding sequence starting from the presumed terminal amino acid of the processed protein codon. The 3' sequence 5' GGGAAGCTTTTAATACTGAAATCGTACAGGAC 3' (SEQ ID NO:4) contains complementary sequences to a Hind III site and is followed by 23 nucleotides of TGF $\alpha$ -HII. The restriction enzyme sites correspond to the restriction enzyme sites on the bacterial expression vector pQE-9 (Qiagen, Inc. Chatsworth, CA, 91311). pQE-9 encodes antibiotic resistance (Amp<sup>r</sup>), a bacterial origin of replication (ori), an IPTG-regulatable promoter operator (P/O), a ribosome binding site (RBS), a 6-His tag and restriction enzyme sites. pQE-9 was then digested with BamHI and HindIII. The amplified sequences were ligated into pQE-9 and were inserted in frame with the sequence encoding for the histidine tag and the RBS. The ligation mixture was then used to transform E. coli strain M15/rep 4 (Qiagen, Inc.) by the procedure described in Sambrook, J. et al., Molecular Cloning: A Laboratory Manual, Cold Spring Laboratory Press, (1989). M15/rep4 contains multiple copies of the plasmid pREP4, which expresses the lacI repressor and also confers kanamycin resistance (Kan<sup>r</sup>). Transformants were identified by their ability to grow on LB plates and ampicillin/kanamycin resistant colonies were selected. Plasmid DNA was isolated



and confirmed by restriction analysis. Clones containing the desired constructs were grown overnight (O/N) in liquid culture in LB media supplemented with both Amp (100 ug/ml) and Kan (25 ug/ml). The O/N culture was used to inoculate a large culture at a ratio of 1:100 to 1:250. The cells were grown to an optical density 600 (O.D.<sup>600</sup>) of between 0.4 and 0.6. IPTG ("Isopropyl-B-D-thiogalacto pyranoside") was then added to a final concentration of 1 mM. IPTG induces by inactivating the lacI repressor, clearing the P/O leading to increased gene expression. Cells were grown an extra 3 to 4 hours. Cells were then harvested by centrifugation. The cell pellet was solubilized in the chaotropic agent 6 Molar Guanidine HCl. After clarification, solubilized TGF $\alpha$ -HII was purified from this solution by chromatography on a Nickel-Chelate column under conditions that allow for tight binding by proteins containing the 6-His tag (Hochuli, E. et al., J. Chromatography 411:177-184 (1984)). TGF $\alpha$ -HII (85 % pure) was eluted from the column in 6 molar guanidine HCl pH 5.0 and for the purpose of renaturation adjusted to 3 molar guanidine HCl, 100mM sodium phosphate, 10 molar glutathione (reduced) and 2 molar glutathione (oxidized). After incubation in this solution for 12 hours the protein was dialyzed to 10 molar sodium phosphate.

#### Example 2

##### Cloning and expression of TGF $\alpha$ -HII using the baculovirus expression system

The DNA sequence encoding the full length TGF $\alpha$ -HII protein, ATCC # \_\_\_\_\_, was amplified using PCR oligonucleotide primers corresponding to the 5' and 3' sequences of the gene:

Three sets of primers were used:

The first set of primers are,

5' CGCGGATCCGCCATCATGCTGCTGTGGGAGTCC 3' (SEQ ID NO:5)  
and 5' GCGTCTAGACTAGTATAGAACACTGTAGTCC 3' (SEQ ID NO:6), this

construct starts at the nucleotide 321 and ends at nucleotide 1248 of SEQ ID NO:1 and includes the putative leader sequence;

The second set of primers are,

5' CGCGGATCCGCCATCATGCTACTCATCGTAGCC 3' (SEQ ID NO:7)

5' GCGTCTAGACTAGTATAGAACACTGTAGTCC 3' (SEQ ID NO:8), this construct starts at nucleotide 402 and ends at nucleotide 1248 of SEQ ID NO:1 and excludes the putative leader sequence;

The third set of primers are,

5' CGCGGATCCAGAACACCACATACCTTGTCCG 3' (SEQ ID NO:9)

5' GCGTCTAGACTAGTATAGAACACTGTAGTCC 3' (SEQ ID NO:10), this construct starts at nucleotide 1100 and ends at nucleotide 1248 of SEQ ID NO:1 and is the putative soluble portion of the polypeptide. All three 5' primers have a BamHI restriction enzyme site (in bold) followed by nucleotides resembling an efficient signal for the initiation of translation in eukaryotic cells (Kozak, M., J. Mol. Biol., 196:947-950 (1987) (the initiation codon for translation "ATG" was underlined). For the third set of primers the baculovirus signal peptide sequence was built in the pA2GP vector.

The 3' primer sequences contain the cleavage site for the restriction endonuclease XbaI and have nucleotides complementary to the 3' TGF $\alpha$  domain of the TGF $\alpha$ -HII gene. The amplified sequences were isolated from a 1% agarose gel using a commercially available kit ("Geneclean," BIO 101 Inc., La Jolla, Ca.). The fragment was then digested with the endonucleases BamHI and XbaI and then purified again on a 1% agarose gel. This fragment was designated F2.

The vector pA2 was used for the first two sets of primers and the pA2GP vector was used with the third set of primers. The pA2 vector (modification of pVL941 vector, discussed below) was used for the expression of the TGF $\alpha$ -HII protein using the baculovirus expression system (for review

see: Summers, M.D. and Smith, G.E. 1987, A manual of methods for baculovirus vectors and insect cell culture procedures, Texas Agricultural Experimental Station Bulletin No. 1555). This expression vector contains the strong polyhedrin promoter of the Autographa californica nuclear polyhedrosis virus (AcMNPV) followed by the recognition sites for the restriction endonucleases. The polyadenylation site of the simian virus (SV)40 was used for efficient polyadenylation. For an easy selection of recombinant virus the beta-galactosidase gene from E.coli was inserted in the same orientation as the polyhedrin promoter followed by the polyadenylation signal of the polyhedrin gene. The polyhedrin sequences were flanked at both sides by viral sequences for the cell-mediated homologous recombination of co-transfected wild-type viral DNA. Many other baculovirus vectors could be used in place of pRG1 such as pAc373, pVL941 and pAcIM1 (Luckow, V.A. and Summers, M.D., Virology, 170:31-39).

The plasmid was digested with the restriction enzymes BamHI and XbaI and then dephosphorylated using calf intestinal phosphatase by procedures known in the art. The DNA was then isolated from a 1% agarose gel using the commercially available kit ("Geneclean" BIO 101 Inc., La Jolla, Ca.). This vector DNA was designated V2.

Fragment F2 and the dephosphorylated plasmid V2 were ligated with T4 DNA ligase. E.coli HB101 cells were then transformed and bacteria identified that contained the plasmid (pBacTGF $\alpha$ -HII) with the TGF $\alpha$ -HII gene using the restriction enzymes BamHI and XbaI. The sequence of the cloned fragment was confirmed by DNA sequencing.

5  $\mu$ g of the plasmid pBacTGF $\alpha$ -HII was co-transfected with 1.0  $\mu$ g of a commercially available linearized baculovirus ("BaculoGold™ baculovirus DNA", Pharmingen, San Diego, CA.) using the lipofection method (Felgner et al. Proc. Natl. Acad. Sci. USA, 84:7413-7417 (1987)).

1  $\mu$ g of BaculoGold™ virus DNA and 5  $\mu$ g of the plasmid pBactGF $\alpha$ -HII were mixed in a sterile well of a microtiter plate containing 50  $\mu$ l of serum free Grace's medium (Life Technologies Inc., Gaithersburg, MD). Afterwards 10  $\mu$ l Lipofectin plus 90  $\mu$ l Grace's medium were added, mixed and incubated for 15 minutes at room temperature. Then the transfection mixture was added drop-wise to the Sf9 insect cells (ATCC CRL 1711) seeded in a 35 mm tissue culture plate with 1 ml Grace's medium without serum. The plate was rocked back and forth to mix the newly added solution. The plate was then incubated for 5 hours at 27°C. After 5 hours the transfection solution was removed from the plate and 1 ml of Grace's insect medium supplemented with 10% fetal calf serum was added. The plate was put back into an incubator and cultivation continued at 27°C for four days.

After four days the supernatant was collected and a plaque assay performed similar as described by Summers and Smith (supra). As a modification an agarose gel with "Blue Gal" (Life Technologies Inc., Gaithersburg) was used which allows an easy isolation of blue stained plaques. (A detailed description of a "plaque assay" can also be found in the user's guide for insect cell culture and baculovirology distributed by Life Technologies Inc., Gaithersburg, page 9-10).

Four days after the serial dilution, the virus was added to the cells and blue stained plaques were picked with the tip of an Eppendorf pipette. The agar containing the recombinant viruses was then resuspended in an Eppendorf tube containing 200  $\mu$ l of Grace's medium. The agar was removed by a brief centrifugation and the supernatant containing the recombinant baculovirus was used to infect Sf9 cells seeded in 35 mm dishes. Four days later the supernatants of these culture dishes were harvested and then stored at 4°C.

Sf9 cells were grown in Grace's medium supplemented with 10% heat-inactivated FBS. The cells were infected with the

recombinant baculovirus V-TGF $\alpha$ -HII at a multiplicity of infection (MOI) of 2. Six hours later the medium was removed and replaced with SF900 II medium minus methionine and cysteine (Life Technologies Inc., Gaithersburg). 42 hours later 5  $\mu$ Ci of  $^{35}$ S-methionine and 5  $\mu$ Ci  $^{35}$ S cysteine (Amersham) were added. The cells were further incubated for 16 hours before they were harvested by centrifugation and the labelled proteins visualized by SDS-PAGE and autoradiography.

### Example 3

#### Expression of Recombinant TGF $\alpha$ -HII in COS cells

The expression of plasmid, TGF $\alpha$ -HII HA was derived from a vector pcDNA3/Amp (Invitrogen) containing: 1) SV40 origin of replication, 2) ampicillin resistance gene, 3) E.coli replication origin, 4) CMV promoter followed by a polylinker region, an SV40 intron and polyadenylation site. A DNA fragment encoding the entire TGF $\alpha$ -HII precursor and a HA tag fused in frame to its 3' end was cloned into the polylinker region of the vector, therefore, the recombinant protein expression was directed under the CMV promoter. The HA tag corresponds to an epitope derived from the influenza hemagglutinin protein as previously described (I. Wilson, H. Niman, R. Heighen, A Cherenson, M. Connolly, and R. Lerner, 1984, Cell 37:767, (1984)). The infusion of HA tag to the target protein allows easy detection of the recombinant protein with an antibody that recognizes the HA epitope.

The plasmid construction strategy was described as follows:

The DNA sequence encoding TGF $\alpha$ -HII, ATCC # \_\_\_\_\_, was constructed by PCR on the original EST cloned using two primers: the 5' primer 5' CGCGGATCCGCCATCATGGTGCTGTGGGAGTCC 3' (SEQ ID NO:11) contains a BamHI site (in bold) followed by 18 nucleotides of TGF $\alpha$ -HII coding sequence starting from the initiation codon; the 3' sequence 5' GCGCTCGAGGTATAGAAC

ACTGTAGTCC 3' (SEQ ID NO:12) contains complementary sequences to an XhoI site, the last 19 nucleotides of the TGF $\alpha$  domain and an XhoI site. The pcDNA3/Amp vector contains BamHI/XhoI cloning sites which bring the PCR insert in frame with the 3' HA tag followed by a stop codon. Therefore, the PCR product contains a BamHI site, 936 base pair coding sequence and an XhoI site. The PCR amplified DNA fragment and the vector, pcDNA3/Amp, were digested with BamHI and XhoI restriction enzyme and ligated. The ligation mixture was transformed into E. coli strain SURE (available from Stratagene Cloning Systems, La Jolla, CA 92037) the transformed culture was plated on ampicillin media plates and resistant colonies were selected. Plasmid DNA was isolated from transformants and examined by restriction analysis for the presence of the correct fragment. For expression of the recombinant TGF $\alpha$ -HII, COS cells were transfected with the expression vector by DEAE-DEXTRAN method (J. Sambrook, E. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, Cold Spring Laboratory Press, (1989)). The expression of the TGF $\alpha$ -HII HA protein was detected by radiolabelling and immunoprecipitation method (E. Harlow, D. Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, (1988)). Cells were labelled for 8 hours with <sup>35</sup>S-cysteine two days post transfection. Culture media was then collected and cells were lysed with detergent (RIPA buffer (150 mM NaCl, 1% NP-40, 0.1% SDS, 1% NP-40, 0.5% DOC, 50mM Tris, pH 7.5) (Wilson, I. et al., Id. 37:767 (1984))). Both cell lysate and culture media were precipitated with an HA specific monoclonal antibody. Proteins precipitated were analyzed on 15% SDS-PAGE gels.

#### Example 4

##### Expression via Gene Therapy

Fibroblasts are obtained from a subject by skin biopsy. The resulting tissue is placed in tissue-culture medium and

separated into small pieces. Small chunks of the tissue are placed on a wet surface of a tissue culture flask, approximately ten pieces are placed in each flask. The flask is turned upside down, closed tight and left at room temperature over night. After 24 hours at room temperature, the flask is inverted and the chunks of tissue remain fixed to the bottom of the flask and fresh media (e.g., Ham's F12 media, with 10% FBS, penicillin and streptomycin, is added. This is then incubated at 37°C for approximately one week. At this time, fresh media is added and subsequently changed every several days. After an additional two weeks in culture, a monolayer of fibroblasts emerge. The monolayer is trypsinized and scaled into larger flasks.

pMV-7 (Kirschmeier, P.T. et al, DNA, 7:219-25 (1988) flanked by the long terminal repeats of the Moloney murine sarcoma virus, is digested with EcoRI and HindIII and subsequently treated with calf intestinal phosphatase. The linear vector is fractionated on agarose gel and purified, using glass beads.

The cDNA encoding a polypeptide of the present invention is amplified using PCR primers which correspond to the 5' and 3' end sequences respectively. The 5' primer containing an EcoRI site and the 3' primer further includes a HindIII site. Equal quantities of the Moloney murine sarcoma virus linear backbone and the amplified EcoRI and HindIII fragment are added together, in the presence of T4 DNA ligase. The resulting mixture is maintained under conditions appropriate for ligation of the two fragments. The ligation mixture is used to transform bacteria HB101, which are then plated onto agar-containing kanamycin for the purpose of confirming that the vector had the gene of interest properly inserted.

The amphotropic pA317 or GP+aml2 packaging cells are grown in tissue culture to confluent density in Dulbecco's Modified Eagles Medium (DMEM) with 10% calf serum (CS), penicillin and streptomycin. The MSV vector containing the

gene is then added to the media and the packaging cells are transduced with the vector. The packaging cells now produce infectious viral particles containing the gene (the packaging cells are now referred to as producer cells).

Fresh media is added to the transduced producer cells, and subsequently, the media is harvested from a 10 cm plate of confluent producer cells. The spent media, containing the infectious viral particles, is filtered through a millipore filter to remove detached producer cells and this media is then used to infect fibroblast cells. Media is removed from a sub-confluent plate of fibroblasts and quickly replaced with the media from the producer cells. This media is removed and replaced with fresh media. If the titer of virus is high, then virtually all fibroblasts will be infected and no selection is required. If the titer is very low, then it is necessary to use a retroviral vector that has a selectable marker, such as neo or his.

The engineered fibroblasts are then injected into the host, either alone or after having been grown to confluence on cytodex 3 microcarrier beads. The fibroblasts now produce the protein product.

Numerous modifications and variations of the present invention are possible in light of the above teachings and, therefore, within the scope of the appended claims, the invention may be practiced otherwise than as particularly described.



## SEQUENCE LISTING

- (1) GENERAL INFORMATION:
- (i) APPLICANT: WEI, ET AL.
- (ii) TITLE OF INVENTION: Transforming Growth Factor  
Alpha HII
- (iii) NUMBER OF SEQUENCES: 12
- (iv) CORRESPONDENCE ADDRESS:
- (A) ADDRESSEE: CARELLA, BYRNE, BAIN, GILFILLAN,  
CECCHI, STEWART & OLSTEIN
- (B) STREET: 6 BECKER FARM ROAD
- (C) CITY: ROSELAND
- (D) STATE: NEW JERSEY
- (E) COUNTRY: USA
- (F) ZIP: 07068
- (v) COMPUTER READABLE FORM:
- (A) MEDIUM TYPE: 3.5 INCH DISKETTE
- (B) COMPUTER: IBM PS/2
- (C) OPERATING SYSTEM: MS-DOS
- (D) SOFTWARE: WORD PERFECT 5.1
- (vi) CURRENT APPLICATION DATA:
- (A) APPLICATION NUMBER:
- (B) FILING DATE:
- (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
- (A) APPLICATION NUMBER: NONE
- (B) FILING DATE: NONE
- (viii) ATTORNEY/AGENT INFORMATION:
- (A) NAME: FERRARO, GREGORY D.
- (B) REGISTRATION NUMBER: 36,134
- (C) REFERENCE/DOCKET NUMBER: 325800-351
- (ix) TELECOMMUNICATION INFORMATION:
- (A) TELEPHONE: 201-994-1700
- (B) TELEFAX: 201-994-1744
- (2) INFORMATION FOR SEQ ID NO:1:
- (i) SEQUENCE CHARACTERISTICS
- (A) LENGTH: 1695 BASE PAIRS
- (B) TYPE: NUCLEIC ACID
- (C) STRANDEDNESS: SINGLE
- (D) TOPOLOGY: LINEAR
- (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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CACTCGTCTG CCCCTGGACT CCCGTCTCCT CCTGTCCTCC GGCTTCCCAG AGCTCCCTCC 60
TTATGGCAGC AGCTTCCCGC GTCTCCGGCG CAGTTCTCAG CGGACGACCC TCTCGCTCCG 120
GGGCTGAGCC CAGTCCCTGG ATGTTGCTGA AACTCTCGAG ATCATGCGCG GGTTCGGCTG 180
CTGCTTCCCC GCCGGGTGCC ACTGCCACCG CCGCCGCTC TGCTGCCGCC GTCCGCGGGA 240
TGCTCAGTAG CCCGCTGCCC GGCCCCCGCG ATCCTGTGTT CCTCGGAAGC CGTTTGCTGC 300
TGCAGAGTTG CACGAAGTAG TCATGGTGCT GTGGGAGTCC CCGCGGCAGT GCAGCAGCTG 360
GACACTTTGC GAGGGCTTTT GCTGGCTGCT GCTGCTGCC GTCATGCTAC TCATCGTAGC 420
CCGCCCCGGT AAGCTCGCTG CTTTCCCTAC CTCCTTAAGT GACTGCCAAA CGCCACCGG 480
CTGGAATTGC TCTGGTTATG ATGACAGAGA AAATGATCTC TTCCTCTGTG ACACCAACAC 540
CTGTAAATTT GATGGGGAAT GTTAAAGAAT TGGAGACACT GTGACTTGCG TCTGTCAGTT 600
CAAGTGCAAC AATGACTATG TGCCTGTGTG TGGCTCCAAT GGGGAGAGCT ACCAGAATGA 660
GTGTTACGCT CGACAGGCTG CATGCAAACA GCAGAGTGAG ATACTTGTGG TGTGAGAAGG 720
ATCATGTGCC ACAGATGCAG GATCAGGATC TGGAGATGGA GTCCATGAAG GCTCTGGAGA 780
AACTAGTCAA AAGGAGACAT CCACCTGTGA TATTTGCCAG TTTGGTGCAG AATGTGACGA 840
AGATGCCGAG GATGTCTGGT GTGTGTGTAA TATTGACTGT TCTCAAACCA ACTTCAATCC 900
CCTCTGCGCT TCTGATGGGA AATCTTATGA TAATGCATGC CAAATCAAAG AAGCATCGTG 960
TCAGAAACAG GAGAAAATTG AAGTCATGTC TTTGGGTGCA TGTCAAGATA ACACAACTAC 1020
AACTACTAAG TCTGAAGATG GGCATTATGC AAGAACAGAT TATGCAGAGA ATGCTAACAA 1080
ATTAGAAGAA AGTGCCAGAG AACACCACAT ACCTTGTCCTG GAACATTACA ATGGCTTCTG 1140
CATGCATGGG AAGTGTGAGC ATTCTATCAA TATCAGGAG CCATCTTGCA GGTGTGATGC 1200
TGTTTAACTG GGACAACACT GTGAAAAAAA GGACTACAGT GTTCTATACG TTGTTCCCGG 1260
TCCTGTACGA TTTCAGTATG TCTTAATCGC AGCTGTGATT GGAACAATTC AGATTGCTGT 1320
CATCTGTGTG GTGGTCTCTT GCATCACAAAG GAAATGCCCC AGAAGCAACA GAATTCACAG 1380
ACAGAAGCAA AATACAGGGC ACTACAGTTC GGACAATACA ACAAGAGCGT CCACGAGGTT 1440
AATCTAAAGG GAGCATGTTT CACAGTGGCT GGACTACCGA GAGCTTGGAC TACACAATAC 1500
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## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 374 AMINO ACIDS  
 (B) TYPE: AMINO ACID  
 (C) STRANDEDNESS:  
 (D) TOPOLOGY: LINEAR

## (ii) MOLECULE TYPE: PROTEIN

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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-30                      -25                      -20
Ile Val Ala Arg Pro Val Lys Leu Ala Ala Phe Pro Thr Ser Leu
-15                      -10                      -5
Ser Asp Cys Gln Thr Pro Thr Gly Trp Asn Cys Ser Gly Tyr Asp
1                      5                      10                      15
Asp Arg Glu Asn Asp Leu Phe Leu Cys Asp Thr Asn Thr Cys Lys
20                      25                      30
Phe Asp Gly Glu Cys Leu Arg Ile Gly Asp Thr Val Thr Cys Val
35                      40                      45
Cys Gln Phe Lys Cys Asn Asn Asp Tyr Val Pro Val Cys Gly Ser
50                      55                      60
Asn Gly Glu Ser Tyr Gln Asn Glu Cys Tyr Leu Arg Gln Ala Ala

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	65		70		75									
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	80				85									90
Ala	Thr	Asp	Ala	Gly	Ser	Gly	Ser	Gly	Asp	Gly	Val	His	Glu	Gly
	95				100									105
Ser	Gly	Glu	Thr	Ser	Gln	Lys	Glu	Thr	Ser	Thr	Cys	Asp	Ile	Cys
	110				115									120
Gln	Phe	Gly	Ala	Glu	Cys	Asp	Glu	Asp	Ala	Glu	Asp	Val	Trp	Cys
	125				130									135
Val	Cys	Asn	Ile	Asp	Cys	Ser	Gln	Thr	Asn	Phe	Asn	Pro	Leu	Cys
	140				145									150
Ala	Ser	Asp	Gly	Lys	Ser	Tyr	Asp	Asn	Ala	Cys	Gln	Ile	Lys	Glu
	155				160									165
Ala	Ser	Cys	Gln	Lys	Gln	Glu	Lys	Ile	Glu	Val	Met	Ser	Leu	Gly
	170				175									180
Arg	Cys	Gln	Asp	Asn	Thr	Thr	Thr	Thr	Thr	Lys	Ser	Glu	Asp	Gly
	185				190									195
His	Tyr	Ala	Arg	Thr	Asp	Tyr	Ala	Glu	Asn	Ala	Asn	Lys	Leu	Glu
	200				205									210
Glu	Ser	Ala	Arg	Glu	His	His	Ile	Pro	Cys	Pro	Glu	His	Tyr	Asn
	215				220									225
Gly	Phe	Cys	Met	His	Gly	Lys	Cys	Glu	His	Ser	Ile	Asn	Met	Gln
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Glu	Pro	Ser	Cys	Arg	Cys	Asp	Ala	Gly	Tyr	Thr	Gly	Gln	His	Cys
	245				250									255
Glu	Lys	Lys	Asp	Tyr	Ser	Val	Leu	Tyr	Val	Val	Pro	Gly	Pro	Val
	260				265									270
Arg	Phe	Gln	Tyr	Val	Leu	Ile	Ala	Ala	Val	Ile	Gly	Thr	Ile	Gln
	275				280									285
Ile	Ala	Val	Ile	Cys	Val	Val	Val	Leu	Cys	Ile	Thr	Arg	Lys	Cys
	290				295									300
Pro	Arg	Ser	Asn	Arg	Ile	His	Arg	Gln	Lys	Gln	Asn	Thr	Gly	His
	305				310									315
Tyr	Ser	Ser	Asp	Asn	Thr	Thr	Arg	Ala	Ser	Thr	Arg	Leu	Ile	
	320				325									

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 30 BASE PAIRS

(B) TYPE: NUCLEIC ACID

(C) STRANDEDNESS: SINGLE

(D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: Oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CCCGGATCCG CACGAGACAT ACCTTGTCGG

30

## (2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS
  - (A) LENGTH: 32 BASE PAIRS
  - (B) TYPE: NUCLEIC ACID
  - (C) STRANDEDNESS: SINGLE
  - (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: Oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GGGAAGCTTT TAATACTGAA ATCGTACAGG AC

32

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS
  - (A) LENGTH: 33 BASE PAIRS
  - (B) TYPE: NUCLEIC ACID
  - (C) STRANDEDNESS: SINGLE
  - (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: Oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CGCGGATCCG CCATCATGGT GCTGTGGGAG TCC

33

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS
  - (A) LENGTH: 31 BASE PAIRS
  - (B) TYPE: NUCLEIC ACID
  - (C) STRANDEDNESS: SINGLE
  - (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: Oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GCGTCTAGAC TAGTATAGAA CACTGTAGTC C

31

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS
  - (A) LENGTH: 33 BASE PAIRS
  - (B) TYPE: NUCLEIC ACID
  - (C) STRANDEDNESS: SINGLE
  - (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: Oligonucleotide

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:  
CGCGGATCCG CCATCATGCT ACTCATCGTA GCC 33
- (2) INFORMATION FOR SEQ ID NO:8:
- (i) SEQUENCE CHARACTERISTICS  
(A) LENGTH: 31 BASE PAIRS  
(B) TYPE: NUCLEIC ACID  
(C) STRANDEDNESS: SINGLE  
(D) TOPOLOGY: LINEAR
- (ii) MOLECULE TYPE: Oligonucleotide
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:  
GCGTCTAGAC TAGTATAGAA CACTGTAGTC C 31
- (2) INFORMATION FOR SEQ ID NO:9:
- (i) SEQUENCE CHARACTERISTICS  
(A) LENGTH: 31 BASE PAIRS  
(B) TYPE: NUCLEIC ACID  
(C) STRANDEDNESS: SINGLE  
(D) TOPOLOGY: LINEAR
- (ii) MOLECULE TYPE: Oligonucleotide
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:  
CGCGGATCCA GAACACCACA TACCTTGTCC G 31
- (2) INFORMATION FOR SEQ ID NO:10:
- (i) SEQUENCE CHARACTERISTICS  
(A) LENGTH: 31 BASE PAIRS  
(B) TYPE: NUCLEIC ACID  
(C) STRANDEDNESS: SINGLE  
(D) TOPOLOGY: LINEAR
- (ii) MOLECULE TYPE: Oligonucleotide
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:  
GCGTCTAGAC TAGTATAGAA CACTGTAGTC C 31
- (2) INFORMATION FOR SEQ ID NO:11:
- (i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 33 BASE PAIRS
- (B) TYPE: NUCLEIC ACID
- (C) STRANDEDNESS: SINGLE
- (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: Oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CGCGGATCCG CCATCATGGT GCTGTGGGAG TCC

33

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS
  - (A) LENGTH: 28 BASE PAIRS
  - (B) TYPE: NUCLEIC ACID
  - (C) STRANDEDNESS: SINGLE
  - (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: Oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GCGCTCGAGG TATAGAACAC TGTAGTCC

28

WHAT IS CLAIMED IS:

1. An isolated polynucleotide comprising a member selected from the group consisting of:
  - (a) a polynucleotide encoding the polypeptide as set forth in SEQ ID NO:2;
  - (b) a polynucleotide encoding the polypeptide comprising amino acids 1 to 329 of SEQ ID NO:2;
  - (c) a polynucleotide encoding the polypeptide comprising amino acids 1 to 264 of SEQ ID NO:2;
  - (d) a polynucleotide encoding the polypeptide comprising amino acids 215 to 329 of SEQ ID NO:2;
  - (e) a polynucleotide encoding the polypeptide comprising amino acids 215 to 264 of SEQ ID NO:2; and
  - (f) a polynucleotide capable of hybridizing to and which is at least 70% identical to the polynucleotide of (a), (b), (c), (d) or (e); and
  - (h) a polynucleotide fragment of the polynucleotide of (a), (b), (c), (d), (e) or (f).
2. The polynucleotide of Claim 1 wherein the polynucleotide is DNA.
3. The polynucleotide of Claim 1 wherein the polynucleotide is RNA.
4. The polynucleotide of Claim 1 wherein the polynucleotide is genomic DNA.
5. The polynucleotide of Claim 2 encoding the polypeptide as set forth in SEQ ID NO:2.
6. The polynucleotide of Claim 2 encoding the polypeptide comprising amino acids 1 to 329 as set forth in SEQ ID NO: 2.

7. The polynucleotide of Claim 2 encoding the polypeptide comprising amino acids 1 to 264 of SEQ ID NO:2.
8. The polynucleotide of Claim 2 encoding the polypeptide comprising amino acids 215 to 329 of SEQ ID NO:2.
9. The polynucleotide of Claim 2 encoding the polypeptide comprising amino acids 215 to 264 of SEQ ID NO:2.
10. An isolated polynucleotide comprising a member selected from the group consisting of:
  - (a) a polynucleotide which encodes a mature polypeptide having the amino acid sequence expressed by the DNA contained in ATCC Deposit No. \_\_\_\_\_;
  - (b) a polynucleotide capable of hybridizing to and which is at least 70% identical to the polynucleotide of (a); and
  - (c) a polynucleotide fragment of the polynucleotide of (a) or (b).
11. The polynucleotide of Claim 1 having the sequence as set forth in SEQ ID NO:1.
12. The polynucleotide of Claim 1 comprising nucleotide 321 to nucleotide 1248 as set forth in SEQ ID NO:1.
13. The polynucleotide of Claim 1 comprising nucleotide 402 to nucleotide 1248 as set forth in SEQ ID NO:1.
14. The polynucleotide of Claim 1 comprising nucleotide 1100 to nucleotide 1248 as set forth in SEQ ID NO:1.



15. A vector containing the DNA of Claim 2.
16. A host cell transformed or transfected with the vector of Claim 15.
17. A process for producing a polypeptide comprising: expressing from the host cell of Claim 16 the polypeptide encoded by said DNA.
18. A process for producing cells capable of expressing a polypeptide comprising genetically engineering cells with the vector of Claim 15.
19. A polypeptide comprising a member selected from the group consisting of:
- (a) a polypeptide having the deduced amino acid sequence of SEQ ID NO: 2;
  - (b) a polypeptide comprising amino acids 1 to 329 as set forth in SEQ ID NO:2;
  - (c) a polypeptide comprising amino acids 215 to 329 as set forth in SEQ ID NO:2;
  - (d) a polypeptide comprising amino acids 215 to 264 as set forth in SEQ ID NO:2;
  - (e) a polypeptide comprising amino acids 1 to 264 as set forth in SEQ ID NO:2;
  - (f) fragments, analogs and derivatives of the polypeptide of (a), (b), (c), (d) or (e); and
  - (g) a polypeptide encoded by the cDNA of ATCC Deposit No. \_\_\_\_\_ and fragments, analogs and derivatives of said polypeptide.
20. The polypeptide of claim 19 comprising amino acid 215 to amino acid 264 of SEQ ID NO:2.
21. An antibody against the polypeptide of claim 19.

22. A compound which inhibits activation of the polypeptide of claim 19.
23. A compound which activates the polypeptide of claim 19.
24. A method for the treatment of a patient having need of TGF $\alpha$ -HII comprising: administering to the patient a therapeutically effective amount of the polypeptide of claim 19.
25. A method for the treatment of a patient having need to inhibit TGF $\alpha$ -HII comprising: administering to the patient a therapeutically effective amount of the compound of Claim 22.
26. The method of Claim 24 wherein said therapeutically effective amount of the polypeptide is administered by providing to the patient DNA encoding said polypeptide and expressing said polypeptide *in vivo*.
27. A process for identifying compounds active as agonists to the polypeptide of Claim 19 comprising:  
contacting a reaction mixture containing a cell type which expresses a TGF $\alpha$ -HII receptor and a compound to be screened; and  
determining if the compound generates a signal from said receptor to identify if the compound is an effective agonist.
28. A process for identifying compounds active as antagonists to the polypeptide of Claim 19 comprising:  
contacting a reaction mixture containing a cell type which expresses the TGF $\alpha$ -HII receptor and a compound to be screened; and

detecting the absence of a signal generated from said receptor after binding of said compound to identify if the compound is an effective antagonist.

29. A process for diagnosing a disease or a susceptibility to a disease comprising:  
determining a mutation in the polynucleotide of claim 1.
30. A diagnostic process comprising:  
analyzing for the presence of the polypeptide of Claim 19 in a sample derived from host.

## FIG. 1A

-320                    -300                    -280  
 CACTCGTCTGCCCTGGACTCCCGTCTCCTCCTGTCTCCTCCGGCTTCCCAGAGCTCCCTCC  
 +-----+-----+-----+-----+-----+-----+-----+-----+  
 GTGAGCAGACGGGACCTGAGGCGACAGAGGAGGACAGGAGGCCGAAGGCTCTCGAGGGAGG  
 -260                    -240                    -220  
 .                    .                    .                    .                    .  
 TTATGGCAGCAGCTTCCCGCGTCTCCGGCGCAGTTCTCAGCGGACGACCCCTCTCGGTCGG  
 +-----+-----+-----+-----+-----+-----+-----+-----+  
 AATACCGTCGTCGAGGCGCAGAGGCCGCGTCAAGAGTCGCCCTGCTGGGAGAGCGAGGC  
 -200                    -180                    -160  
 .                    .                    .                    .                    .  
 GGGCTGAGCCCAGTCCCTGGATGTTGCTGAAACTCTCGAGATCATGCGCGGTTTGGCTG  
 +-----+-----+-----+-----+-----+-----+-----+-----+  
 CCCGACTCGGGTCAGGACCTACAACGACTTTGAGAGCTCTAGTACGCGCCCCAAACCGAC  
 -140                    -120                    -100  
 .                    .                    .                    .                    .  
 CTGCTTCCCCCGGGTGCCCACTGCCACCGCCGCCCTCTGCTGCCGCCGTCCGGCGGA  
 +-----+-----+-----+-----+-----+-----+-----+-----+  
 GACGAAGGGCGGCCACGGTGACGGTGGCGGCGGGGAGACGACGGCGGCGAGGGCCCT  
 -80                    -60                    -40  
 .                    .                    .                    .                    .  
 TGCTCAGTAGCCCGCTGCCCGGCCCGCGATCCTGTGTCTCCTCGAAGCCGTTTGTCTGC  
 +-----+-----+-----+-----+-----+-----+-----+-----+  
 ACCAGTCATCGGGCGACGGCGCGGGCGGCTAGGACACAGGAGCCCTTCGGCAACGACG  
 -20                    0                    20  
 MATCH WITH FIG. 1B

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GACACTTTCGAGGGCTTTTGCTGGCTGCTGCTGCCCGTCATGCTACTCATCGTAGC  
-----+-----+-----+-----+-----+-----+  
CTGTGAACGCTCCGAAACGACGACGACGACGACGGGCAGTACGATGAGTAGCATCG  
T L C E G F C W L L L L L P V M L L I V A  
100 120 140

CGGCCGGTGAAGCTCGCTGCTTCCCTACCTCCTTAAGTGAAGTCCAAACGCCACCGG  
 +-----+-----+-----+-----+-----+-----+-----+-----+  
 GGCGGCCACTTCGAGCGACGAAAGGATGGAGGAATTCACGTGACGGTTGCGGGTGCGC  
 R P V K L A A F P T S L S D C Q T P T G  
 160 180 200

CTGGAATTGCTCTGGTTATGATGACAGAGAAAATGATCTCTTCCCTCTGTGACACCAACAC  
-----+-----+-----+-----+-----+-----+-----  
GACCTTAACGAGACCAATACTACTGTCTCTTTTACTAGAGAAGGAGACACTGTGGTTGTG  
W N C S G Y D D R E N D L F L C D T N T  
220 240 260

CTGTAAATTTCGGGAATGTTTAAGAAATTCGACACACTGTGACTTGGCTCTGTCAGTT  
MATCH WITH FIG. 1C

FIG. 1C

MATCH WITH FIG. 1B

GACATTAACTACCCCTTACAAATTCTTAACCTCTGTGACACTGAACGCAGACAGTCAA  
 C K F D G E C L R I G D T V T C V C Q F

280 300 320

CAAGTCCAACAATGACTATGTGCCCTGTGTGGCTCCAATGGGAGAGCTACCAGAAATGA

GTTCACGTTGTTACTGATACACGACACACACCGAGGTTACCCCTCTCGATGGTCTTACT  
 K C N N D Y V P V C G S N G E S Y Q N E  
 340 360 380

GTGTTACCTGCAGCGCTGCCATGCAACAGCAGTGAGATACTTGGTGTCTAGAAGG  
-----+-----+-----+-----+-----+-----  
CACAAATGGACGCTGTCCGACGTACGTTGTTCGTCTCACTCTATGAACACCACAGTCTTCC  
C Y L R Q A A C K Q Q S E I L V V S E G  
400 420 440

ATCATGTGCCACAGATGCAGGATCAGGATCTGGAGATGGAGTCCATGAAGCTCTCTGGAGA  
 TAGTACACGGTGTACGTCTAGTCTAGACCTCTACCTCAGTACTTCCGAGACCTCT  
 S C A T D A G S G S G D G V H E G S G E  
 460 480 500

AACTAGTCAAAGGAGACATCCACCTGTGATAATTGCCAGTTTGCGTCAGAAATGTGACGA  
-+-----+-----+-----+-----+-----+-----+-----+-----  
TTTGATCAGTTTTCTCTGTAGTGGACACTATAAACGGTCAAAACCACGTCTTACACTGCT

MATCH WITH FIG. 1D

MATCH WITH FIG. 1D



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## FIG. 1E

MATCH WITH FIG. 1D

ATTAGAAGAAAGTCCAGAGAACACCACATACCTTGTCGGAACATTACAATGGCTTCTG  
 TAATCTTCTTTACCGTCTCTGTGGTGATGGAACAGGCCCTTGTAATGTTACCGAAGAC  
 L E S A R E H H I P C P E H Y N G F C  
 820 840 860

CATGCATGGGAAGTGTGAGCATTCTATCAATATGCAGGAGCCATCTTGCAGGTGATGC  
 GTACGTACCCCTTCACACTCGTAAGATAGTTATACGTCCTCGGTAGAACGTCCACACTACG  
 M H G K C E H S I N M Q E P S C R C D A  
 880 900 920  
 TGGTTATACTGGACAACACTGTGAAAAAAGGACTACAGTGTCTTATACGTTGTTCCCGG  
 ACCAATATGACCTGTTGTGACACTTTTTTCCCTGATGTCACAAGATATGCAACAAGGGCC  
 G Y T G Q H C E K K D Y S V L Y V V P G  
 940 960 980

TCCTGTACGATTTCAGTATGTCTTAATCGCAGCTGTGATTGGAACAATTCAGATTGCTGT  
 AGGACATGCTAAAGTCATACAGAAATTAGCGTCGACACTAACCTTGTTAAGTCTAACGACA  
 P V R F Q Y V L I A A V I G T I Q I A V  
 1000 1020 1040  
 CATCTGTGTGGTCCCTCTGCATCACAGGAATGCCCCAGAACAGAAATTCACAG  
 GTAGACACACCAGGAGACGTAGTGTCCCTTTACGGGGTCTTCGTTGTCTTAAGTGTC  
 MATCH WITH FIG. 1F

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## MATCH WITH FIG. 1E

```

I  C  V  V  V  L  C  I  T  R  K  C  P  R  S  N  R  I  H  R
1060      .      1080      .      1100
ACAGAAGCAAATACAGGGCACTACAGTTCGGACAATAACAAGAGCGTCCACGAGGTT
+-----+-----+-----+-----+-----+-----+-----+
TGTCCTTCGTTTATGTCCCGTGATGTCAAGCCTGTTATGTTGTTCTCGCAGGTGCTCCAA
Q  K  Q  N  T  G  H  Y  S  S  D  N  T  T  R  A  S  T  R  L
1120      .      1140      .      1160
AATCTAAAGGGAGCATGTTTTCACAGTGGCTGGACTACCGAGAGCTTGGACTACACAATAC
+-----+-----+-----+-----+-----+-----+-----+
TTAGATTTCCCTCGTACAAAGTGTCAACCGACCTGATGGCTCTCGAACCTGATGTTATG
I  *
1180      .      1200      .      1220
AGTATTATAGACAAAGAATAAGACAAGAGATCTACACATGTTGCCCTTGCAATTGTGTA
+-----+-----+-----+-----+-----+-----+-----+
TCATAATATCIGTTTTCTTATTCTGTTCTCTAGATGTGTACAACGGAACGTAACACCAT
1240      .      1260      .      1280
ATCTACACCAATGAAACAATGTACTACAGCTATATTTGATTATGTATGGATATATTGAA
+-----+-----+-----+-----+-----+-----+-----+
TAGATGTGGTTACTTTTGTACATGATGTGCGATATAAACTAATACATACCTATATAAAGTT
1300      .      1320      .      1340
ATAGTATACATTGTCTTGATGTTTTTCTGTAATGTAATAAATACTATTTATATCACACAA
+-----+-----+-----+-----+-----+-----+-----+
TATCATATGTAACAGAACTACAAAAAAGACATTACATTTTATTGATAAATATAGTGTGTT
1360
AAAAA
+-----+-----+-----+-----+-----+-----+-----+
TTTTTTTTTTTTTTTT

```

FIG. 1F

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	10	20	30	Majority
1	M D	R A A R C S G	A S S L P L L L A L A L G	hBCp
11	M V	- - - - - P S	A G Q L A L F - - - A L G	hTGFap
1	M V	L W E S P R Q C S S W T L C E G F C W L L L P V M L L	T G F a 9 5 . a a	
	I V	- - - - - L X X C Q X X X X X X - -	Majority	
	40	50	60	
23	L V	- - - - - I L H C V V A D G N S T R S - -	hBCp	
15	I V	- - - - - L A A C Q A L E N S T S P - -	hTGFap	
31	I V	A R P V K L A A F P T S L S D C Q T P T G W N C S G Y D	T G F a 9 5 . a a	
	- X E X X X L C X D - - - - -	Majority		
	70	80	90	
39	- P E T N G L L C G D - - - - -	- - - - -	- - - - -	hBCp
30	- - - - - L S A D - - - - -	- - - - -	- - - - -	hTGFap
61	D R E N D L F L C D T N T C K F D G E C L R I G D T V T C V	T G F a 9 5 . a a		
	- - - - -	Majority		
	100	110	120	
49	- - - - -	- - - - -	- - - - -	hBCp
34	- - - - -	- - - - -	- - - - -	hTGFap

**SUBSTITUTE SHEET (RULE 26)**

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MATCH WITH FIG. 2A		FIG. 2B	
91	C Q F K C N N D Y V P V C G S N G E S Y Q N E C Y L R Q A A	TGFa95.aa	
	- - - - - P E X X C A X - - - - -	Majority	
	130	140	150
49	- - - - - P E N C A A - - - - -	hBCp	
34	- - - - - P P V A A V - - - - -	hTGFap	
121	C K Q Q S E I L V S E G S C A T D A G S G S G D G V H E G	TGFa95.aa	
	- - - - -	Majority	
	160	170	180
56	- - - - -	hBCp	
41	- - - - -	hTGFap	
151	S G E T S Q K E T S T C D I C Q F G A E C D E D A E D V W C	TGFa95.aa	
	- - - - -	Majority	
	190	200	210
56	- - - - -	hBCp	
41	- - - - -	hTGFap	
181	V C N I D C S Q T N F N P L C A S D G K S Y D N A C Q I K E	TGFa95.aa	
	- - - - -	Majority	
	220	230	240
	- - - - - T T T X X X X X G	Majority	
MATCH WITH FIG. 2C			

SUBSTITUTE SHEET (RULE 26)

56	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	T	T	Q	S	K	R	K	G	hBCp	
41	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	V	S	-	-	-	-	-	-	-	htGFap	
211	A	S	C	Q	K	E	K	I	E	V	M	S	L	G	R	C	Q	D	N	T	T	T	T	T	G	TGfa95.aa	
	H	F	X	R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	P	X	X	Y	X	Majority

250 260 270

	H	F	S	R	-	-	-	-	-	-	-	-	-	C	P	K	Q	Y	K	hBCp				
65	H	F	S	R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-				
43	H	F	N	D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-				
241	H	Y	A	R	T	D	Y	A	E	N	A	N	K	L	E	E	S	A	R	E	H	Y	N	TGFA95.aa

X	F	C	X	H	G	X	C	R	F	X	V	X	E	Q	X	P	S	C	V	C	D	X	G	Y	X	G	A	R	C		Majority
---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	--	----------

280 290 300

75  
53  
271

H	Y	C	I	K	G	R	C	R	F	V	A	E	Q	T	P	S	C	V	C	D	E	G	Y	I	G	A	R	C	hBcp
O	F	C	F	H	G	T	C	R	F	V	Q	E	D	P	A	C	C	V	C	H	S	G	Y	V	G	A	R	C	hTGFap
G	F	C	M	H	G	K	C	E	H	S	I	N	M	P	S	C	R	C	D	A	G	Y	T	G	Q	H	C	TGFa95.aa	

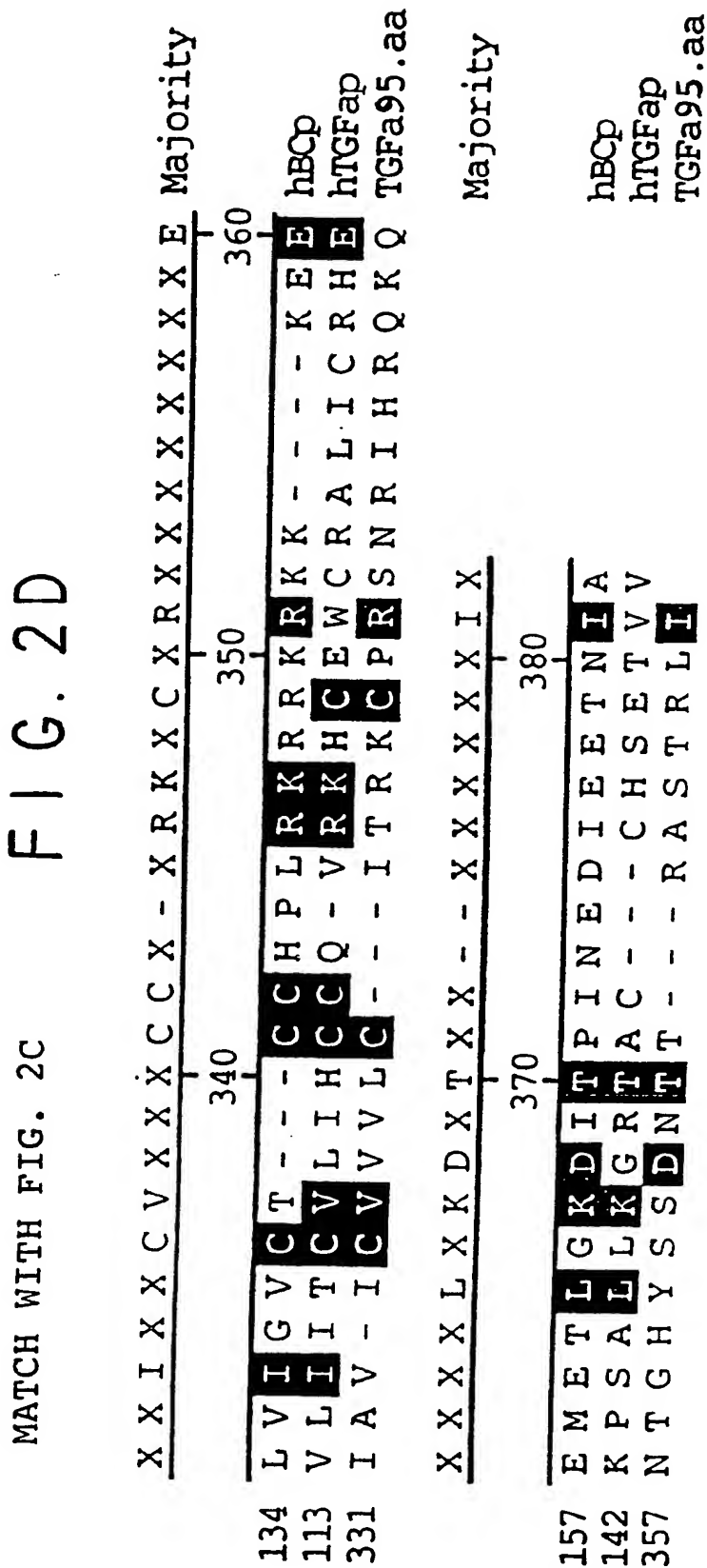
E X X D L X X L X X X G X X X I X X L I A V X I V X I X Majority

310	320	330
-----	-----	-----

105	E R V D L F Y L R G D R G Q I L V I	L I A V M V V F I I	hBQp
83	E H A D L L A V V A S Q K Q A I I	A L V V S I V A L A	htGFap
301	E K'K D Y S V L Y V V P G P V R F Q Y V	L I A V L I A V I G T I Q	TGFa95.aa

MATCH WITH FIG. 2D

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Decoration 'Decoration #1': Shade with solid residues that match the Consensus exactly.

Applicant's or agent's file reference number: <b>325800-351</b>	International application No.: <b>PCT/US95/06386</b>
--	--

# **INDICATIONS RELATING TO A DEPOSITED MICROORGANISM**

(PCT Rule 13bis)

<b>A.</b> The indications made below relate to the microorganism referred to in the description on pages <u>5, 37, 38, 42, 54, and 55</u> lines <u>53, 5, 41, 28, 17, and 34</u> , respectively.	
<b>B. IDENTIFICATION OF DEPOSIT</b> <span style="float: right;">Further deposits are identified on an additional sheet <input type="checkbox"/></span>	
Name of depository institution  <b>American Type Culture Collection</b>	
Address of depository institution (including postal code and country)  <b>12301 Parklawn Drive Rockville, Maryland 20852 U.S.A.</b>	
Date of deposit: <b>May 24, 1995</b>	Accession Number: <b>97160</b>
<b>C. ADDITIONAL INDICATIONS</b> (leave blank if not applicable) <span style="float: right;">This information is contained on an additional sheet <input type="checkbox"/></span>	
<b>D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE</b> (if the indications are not for all designated States)	
<b>E. SEPARATE FURNISHING OF INDICATIONS</b> (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	

For receiving Office use only	For International Bureau use only
<input type="checkbox"/> This sheet was received with the international application	<input type="checkbox"/> This sheet was received by the International Bureau on:
Authorized officer	<b>29 OCT 1996 (29 10 96)</b> Authorized officer <b>Ellen Moyse</b>

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/06386**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : C12N 15/12, 15/18, 15/19; C07K 14/495

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 69.1, 172.3, 240.2, 320.1; 530/324, 350, 399; 536/23.5, 24.3, 24.31, 24.33

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
APS and DIALOG (files 5, 155, 351, 357, 358) search terms: TGF, transforming growth factor, betacellulin, alpha**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US, A, 5,182,261 (TAM) 26 January 1993, see abstract and claims, Figure 1.	1-20
Y	US, A, 5,240,912 (TODARO) 31 August 1993, see abstract, claims, particularly claim 1.	19
-----		-----
A		1-18, 20

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be part of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z*	document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means		
*P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 04 AUGUST 1995	Date of mailing of the international search report 29 AUG 1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer <i>Marianne Porta Allen</i> MARIANNE PORTA ALLEN Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)\*

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/06386

## A. CLASSIFICATION OF SUBJECT MATTER: US CL :

435/6, 69.1, 172.3, 240.2, 320.1; 530/324, 350, 399; 536/23.5, 24.3, 24.31, 24.33

## BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-20, drawn to nucleic acid sequences and the encoded proteins.

Group II, claim 21, drawn to antibodies.

Group III, claim 22, drawn to inhibitory compounds.

Group IV, claim 23, drawn to receptor activating compounds.

Group V, claim 24, drawn to a method of treatment by administering the protein.

Group VI, claim 25, drawn to a method of treatment by administering the inhibitory compound.

Group VII, claim 26, drawn to a method of treatment by administering a nucleic acid sequence encoding the protein.

Group VIII, claims 27-28, drawn to a method for identifying compounds.

Group IX, claim 29, drawn to a method of diagnosis using nucleic acid sequences.

Group X, claim 30, drawn to a method of diagnosis using the protein.

The inventions listed as Groups I-X do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Groups I-IV are drawn to structurally different products which do not share the same or a corresponding special technical feature. Groups V-X are drawn to methods having different goals, method steps, and starting materials which do not share the same or a corresponding special technical feature. Note that PCT Rule 13 does not provide for multiple products or methods within a single application.



# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/06386

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:  
1-20

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1))(July 1992)\*

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